



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b>  <b>G01N 33/543</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 00/16101</b>  <b>(43) International Publication Date:</b> 23 March 2000 (23.03.00)
<b>(21) International Application Number:</b> PCT/US99/20914  <b>(22) International Filing Date:</b> 10 September 1999 (10.09.99)  <b>(30) Priority Data:</b> 09/151,877                      11 September 1998 (11.09.98)    US  <b>(71) Applicant (for all designated States except US):</b> TRUSTEES OF TUFTS COLLEGE [US/US]; 136 Harrison Avenue, Boston, MA 02111 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> WALT, David, R. [US/US]; 4 Candlewick Close, Lexington, MA 02178 (US). MICHAEL, Karri, L. [US/US]; 77 Partridge Avenue, Somerville, MA 02145 (US).  <b>(74) Agents:</b> BREZNER, David, J. et al.; Flehr, Hohbach, Test, Albritton & Herbert LLP, Suite 3400, 4 Embarcadero Center, San Francisco, CA 94111-4187 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished          upon receipt of that report.</i>
<b>(54) Title:</b> TARGET ANALYTE SENSORS UTILIZING MICROSPHERES		
<b>(57) Abstract</b>  A microsphere-based analytic chemistry system and method for making the same is disclosed in which microspheres or particles carrying bioactive agents may be combined randomly or in ordered fashion and dispersed on a substrate to form an array while maintaining the ability to identify the location of bioactive agents and particles within the array using an optically interrogatable, optical signature encoding scheme. A wide variety of modified substrates may be employed which provide either discrete or non-discrete sites for accommodating the microspheres in either random or patterned distributions. The substrates may be constructed from a variety of materials to form either two-dimensional or three-dimensional configurations. In a preferred embodiment, a modified fiber optic bundle or array is employed as a substrate to produce a high density array. The disclosed system and method have utility for detecting target analytes and screening large libraries of bioactive agent.		

## TARGET ANALYTE SENSORS UTILIZING MICROSPHERES

5 This application is a continuation-in-part of copending application U.S.S.N. 08/818,919, filed March 14, 1997.

## BACKGROUND OF THE INVENTION

10 The use of optical fibers and optical fiber strands in combination with light absorbing dyes for chemical analytical determinations has undergone rapid development, particularly within the last decade. The use of optical fibers for such purposes and techniques is described by Milanovich et al., "Novel Optical Fiber Techniques For Medical Application", Proceedings of the SPIE 28th Annual International Technical Symposium On Optics and Electro-Optics, Volume 494, 1980; Seitz, W.R., "Chemical Sensors Based On Immobilized Indicators and Fiber Optics" in *C.R.C. Critical Reviews In Analytical Chemistry*, Vol. 19, 1988, pp. 135-173; Wolfbeis, O.S., "Fiber Optical Fluorosensors In Analytical Chemistry" in *Molecular Luminescence Spectroscopy, Methods and Applications* (S. G. Schulman, editor), Wiley & Sons, New York (1988); Angel, S.M., *Spectroscopy* 2 (4):38 (1987); Walt, et al., 15 "Chemical Sensors and Microinstrumentation", *ACS Symposium Series*, Vol. 403, 1989, p. 252, and Wolfbeis, O.S., *Fiber Optic Chemical Sensors*, Ed. CRC Press, Boca Raton, FL, 1991, 2nd Volume.

20 When using an optical fiber in an *in vitro/in vivo* sensor, one or more light absorbing dyes are located near its distal end. Typically, light from an appropriate source is used to illuminate the dyes through the fiber's proximal end. The light propagates along the length of the optical fiber; and a portion of this propagated light exits the distal end and is absorbed by the dyes. The light absorbing dye may or may not be immobilized; may or may not be directly attached to the optical fiber itself; may or may not be suspended in a fluid sample containing one or more analytes of interest; and may or may not be retainable for subsequent use in a second optical determination.

25 Once the light has been absorbed by the dye, some light of varying wavelength and intensity returns, conveyed through either the same fiber or collection fiber(s) to a detection system where it is observed

and measured. The interactions between the light conveyed by the optical fiber and the properties of the light absorbing dye provide an optical basis for both qualitative and quantitative determinations.

Of the many different classes of light absorbing dyes which conventionally are employed with bundles of fiber strands and optical fibers for different analytical purposes are those more common compositions that emit light after absorption termed "fluorophores" and those which absorb light and internally convert the absorbed light to heat, rather than emit it as light, termed "chromophores."

Fluorescence is a physical phenomenon based upon the ability of some molecules to absorb light (photons) at specified wavelengths and then emit light of a longer wavelength and at a lower energy. Substances able to fluoresce share a number of common characteristics: the ability to absorb light energy at one wavelength  $\lambda_{ab}$ ; reach an excited energy state; and subsequently emit light at another light wavelength,  $\lambda_{em}$ . The absorption and fluorescence emission spectra are individual for each fluorophore and are often graphically represented as two separate curves that are slightly overlapping. The same fluorescence emission spectrum is generally observed irrespective of the wavelength of the exciting light and, accordingly, the wavelength and energy of the exciting light may be varied within limits; but the light emitted by the fluorophore will always provide the same emission spectrum. Finally, the strength of the fluorescence signal may be measured as the quantum yield of light emitted. The fluorescence quantum yield is the ratio of the number of photons emitted in comparison to the number of photons initially absorbed by the fluorophore. For more detailed information regarding each of these characteristics, the following references are recommended: Lakowicz, J. R., *Principles of Fluorescence Spectroscopy*, Plenum Press, New York, 1983; Freifelder, D., *Physical Biochemistry*, second edition, W. H. Freeman and Company, New York, 1982; "Molecular Luminescence Spectroscopy Methods and Applications: Part I" (S.G. Schulman, editor) in *Chemical Analysis*, vol. 77, Wiley & Sons, Inc., 1985; *The Theory of Luminescence*, Stepanov and Gribkovskii, Hiffe Books, Ltd., London, 1968.

In comparison, substances which absorb light and do not fluoresce usually convert the light into heat or kinetic energy. The ability to internally convert the absorbed light identifies the dye as a "chromophore." Dyes which absorb light energy as chromophores do so at individual wavelengths of energy and are characterized by a distinctive molar absorption coefficient at that wavelength. Chemical analysis employing fiber optic strands and absorption spectroscopy using visible and ultraviolet light wavelengths in combination with the absorption coefficient allow for the determination of concentration for specific analyses of interest by spectral measurement. The most common use of absorbance measurement via optical fibers is to determine concentration which is calculated in accordance with Beers' law; accordingly, at a single absorbance wavelength, the greater the quantity of the composition which absorbs light energy at a given wavelength, the greater the optical density for

the sample. In this way, the total quantity of light absorbed directly correlates with the quantity of the composition in the sample.

Many of the recent improvements employing optical fiber sensors in both qualitative and quantitative analytical determinations concern the desirability of depositing and/or immobilizing various light absorbing dyes at the distal end of the optical fiber. In this manner, a variety of different optical fiber chemical sensors and methods have been reported for specific analytical determinations and applications such as pH measurement, oxygen detection, and carbon dioxide analyses. These developments are exemplified by the following publications: Freeman, et al., *Anal. Chem.* 53:98 (1983); Lippitsch et al., *Anal. Chem. Acta.* 205:1, (1988); Wolfbeis et al., *Anal. Chem.* 60:2028 (1988); Jordan, et al., *Anal. Chem.* 59:437 (1987); Lubbers et al., *Sens. Actuators* 1983; Munkholm et al., *Talanta* 35:109 (1988); Munkholm et al., *Anal. Chem.* 58:1427 (1986); Seitz, W. R., *Anal. Chem.* 56:16A-34A (1984); Peterson, et al., *Anal. Chem.* 52:864 (1980); Saari, et al., *Anal. Chem.* 54:821 (1982); Saari, et al., *Anal. Chem.* 55:667 (1983); Zhujun et al., *Anal. Chem. Acta.* 160:47 (1984); Schwab, et al., *Anal. Chem.* 56:2199 (1984); Wolfbeis, U.S., "Fiber Optic Chemical Sensors", Ed. CRC Press, Boca Raton, FL, 1991, 2nd Volume; and Pantano, P., Walt, D.R., *Anal. Chem.*, 481A-487A, Vol. 67, (1995).

More recently, fiber optic sensors have been constructed that permit the use of multiple dyes with a single, discrete fiber optic bundle. U.S. Pat. Nos. 5,244,636 and 5,250,264 to Walt, et al. disclose systems for affixing multiple, different dyes on the distal end of the bundle, the teachings of each of these patents being incorporated herein by this reference. The disclosed configurations enable separate optical fibers of the bundle to optically access individual dyes. This avoids the problem of deconvolving the separate signals in the returning light from each dye, which arises when the signals from two or more dyes are combined, each dye being sensitive to a different analyte, and there is significant overlap in the dyes' emission spectra.

The innovation of the two previous patents was the placement of multiple chemical functionalities at the end of a single optical fiber bundle sensor. This configuration yielded an analytic chemistry sensor that could be remotely monitored via the typically small bundle. The drawback, however, was the difficulty in applying the various chemistries associated with the chemical functionalities at the sensor's end; the functionalities were built on the sensor's end in a serial fashion. This was a slow process, and in practice, only tens of functionalities could be applied. Accordingly, compositions and methods are desirable that allow the generation of large fiber optic arrays including microspheres that can be either encoded or decoded to allow the detection of target analytes.

#### SUMMARY OF THE INVENTION



In accordance with the above objects, the present invention provides compositions comprising a substrate with a surface comprising discrete sites, and a population of microspheres distributed on the sites. The sites may be wells or chemically functionalized sites.

5 In an additional aspect the invention provides methods of determining the presence of a target analyte in a sample. The methods comprise contacting the sample with a composition comprising a substrate with a surface comprising discrete sites, and a population of microspheres comprising at least a first and a second subpopulation. Each subpopulation comprises a bioactive agent and an optical signature capable of identifying the bioactive agent. The microspheres are distributed on the surface such that the discrete sites contain microspheres. The presence or absence of the target analyte is  
10 then determined.

In a further aspect, the invention provides methods of making a composition comprising forming a surface comprising individual sites on a substrate, distributing microspheres on the surface such that the individual sites contain microspheres. The microspheres comprise at least a first and a second subpopulation, each comprising a bioactive agent, and an optical signature capable of identifying said  
15 bioactive agent.

#### BRIEF DESCRIPTION OF THE DRAWINGS

In the accompanying drawings, reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale; emphasis has instead been placed upon illustrating the principles of the invention. Of the drawings:

20 Fig. 1 is a schematic diagram illustrating the optical signature encoding and chemical functionalizing of the microspheres according to the present invention;

Fig. 2 is a process diagram describing the preparation, encoding, and functionalizing of the microspheres of the present invention;

25 Fig. 3 is a schematic diagram illustrating a microsphere system including microspheres with different chemical functionalities and encoded descriptions of the functionalities;

Fig. 4 is a schematic diagram of the inventive fiber optic sensor and associated instrumentation and control system;

Fig. 5A and 5B are micrographs illustrating the preferred technique for attaching or affixing the microspheres to the distal end of the optical fiber bundle;

Fig. 6 is a process diagram describing well formation in the optical fiber bundle and affixation of the microspheres in the wells;

5 Figs. 7A and 7B are micrographs showing the array of microspheres in their corresponding wells prior and subsequent to physical agitation, tapping and air pulsing, demonstrating the electrostatic binding of the microspheres in the wells;

10 Figs. 8A, 8B, and 8C are micrographs from alkaline phosphatase microspheres when exposed to fluorescein diphosphate, at the fluorescein emission wavelength, at an encoding wavelength for DilC, and at an encoding wavelength for TRC, respectively;

Figs. 9A and 9B are micrographs showing the optical signal from  $\beta$ -galactosidase microspheres when exposed to fluorescein  $\beta$ -galactopyranoside at the fluorescein emission wavelength and at an encoding wavelength for DilC, respectively; and

15 Fig. 10A and 10B are micrographs showing the optical response from rabbit antibody microspheres prior to and post, respectively, exposure to fluorescein labeled antigens.

20 Fig. 11A and 11B are micrographs depicting the optical response from beads synthesized with DNA on the bead surface, following a 10 min. hybridization with a Cy3-labeled probe complementary to the sequence of the DNA immobilized on the bead. Beads were randomly distributed on A) an etched optical imaging fiber or B) a patterned polymer (polyurethane) substrate (a chip). Following hybridization with 5 nM Cy3-labeled probe, the substrates were placed in buffer for optical readout on an imaging system. A) was imaged through the proximal end, with the distal (beaded) end in buffer solution. B) was imaged directly from the top, through a coverslip.

25 Fig. 12A, 12B and 12C are micrographs depicting the optical responses between different substrates. The substrate in A) and B) is an etched optical imaging fiber, and the substrate in C) is a chip. Data were obtained as described in Fig 11, and quantified to determine mean intensity and variability.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is based on two synergistic inventions: 1) the development of a bead-based analytic chemistry system in which beads, also termed microspheres, carrying different chemical

functionalities may be mixed together while the ability is retained to identify the functionality of each bead using an optically interrogatable encoding scheme (an "optical signature"); and 2) the use of a substrate comprising a patterned surface containing individual sites that can bind or associate individual beads. This allows the synthesis of the bioactive agents (i.e. compounds such as nucleic acids and antibodies) to be separated from their placement on an array, i.e. the bioactive agents may be synthesized on the beads, and then the beads are randomly distributed on a patterned surface. Since the beads are first coded with an optical signature, this means that the array can later be "decoded", i.e. after the array is made, a correlation of the location of an individual site on the array with the bead or bioactive agent at that particular site can be made. This means that the beads may be randomly distributed on the array, a fast and inexpensive process as compared to either the *in situ* synthesis or spotting techniques of the prior art. Once the array is loaded with the beads, the array can be decoded, or can be used, with full or partial decoding occurring after testing, as is more fully outlined below.

Accordingly, the present invention provides array compositions comprising at least a first substrate with a surface comprising individual sites. By "array" herein is meant a plurality of bioactive agents in an array format; the size of the array will depend on the composition and end use of the array. Arrays containing from about 2 different bioactive agents (i.e. different beads) to many millions can be made, with very large fiber optic arrays being possible. Generally, the array will comprise from two to as many as a billion or more, depending on the size of the beads and the substrate, as well as the end use of the array, thus very high density, high density, moderate density, low density and very low density arrays may be made. Preferred ranges for very high density arrays are from about 10,000,000 to about 2,000,000,000, with from about 100,000,000 to about 1,000,000,000 being preferred. High density arrays range about 100,000 to about 10,000,000, with from about 1,000,000 to about 5,000,000 being particularly preferred. Moderate density arrays range from about 10,000 to about 50,000 being particularly preferred, and from about 20,000 to about 30,000 being especially preferred. Low density arrays are generally less than 10,000, with from about 1,000 to about 5,000 being preferred. Very low density arrays are less than 1,000, with from about 10 to about 1000 being preferred, and from about 100 to about 500 being particularly preferred. In some embodiments, the compositions of the invention may not be in array format; that is, for some embodiments, compositions comprising a single bioactive agent may be made as well. In addition, in some arrays, multiple substrates may be used, either of different or identical compositions. Thus for example, large arrays may comprise a plurality of smaller substrates.

In addition, one advantage of the present compositions is that particularly through the use of fiber optic technology, extremely high density arrays can be made. Thus for example, because beads of 200 nm can be used, and very small fibers are known, it is possible to have as many as 250,000 different

fibers and beads in a 1 mm<sup>2</sup> fiber optic bundle, with densities of greater than 15,000,000 individual beads and fibers per 0.5 cm<sup>2</sup> obtainable.

5 The compositions comprise a substrate. By "substrate" or "solid support" or other grammatical equivalents herein is meant any material that can be modified to contain discrete individual sites appropriate for the attachment or association of beads and is amenable to at least one detection method. As will be appreciated by those in the art, the number of possible substrates are very large, and include, but are not limited to, glass and modified or functionalized glass, plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, Teflon, etc.), polysaccharides, nylon or nitrocellulose, resins, silica or 10 silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses, plastics, optical fiber bundles, and a variety of other polymers. In general, the substrates allow optical detection and do not appreciably fluoresce.

In one embodiment, the substrate does not comprise an optical fiber bundle or array.

15 Generally the substrate is planar, although as will be appreciated by those in the art, other configurations of substrates may be used as well; for example, three dimensional configurations can be used, for example by embedding the beads in a porous block of plastic that allows sample access to the beads and using a confocal microscope for detection. Similarly, the beads may be placed on the inside surface of a tube, for flow-through sample analysis to minimize sample volume. Preferred substrates include optical fiber bundles as discussed below, and flat planar substrates such as glass, 20 polystyrene and other plastics and acrylics.

At least one surface of the substrate is modified to contain discrete, individual sites for later association of microspheres. These sites may comprise physically altered sites, i.e. physical configurations such as wells or small depressions in the substrate that can retain the beads, such that a microsphere can rest in the well, or the use of other forces (magnetic or compressive), or chemically 25 altered or active sites, such as chemically functionalized sites, electrostatically altered sites, hydrophobically/ hydrophilically functionalized sites, spots of adhesive, etc.

The sites may be a pattern, i.e. a regular design or configuration, or randomly distributed. A preferred embodiment utilizes a regular pattern of sites such that the sites may be addressed in the X-Y coordinate plane. "Pattern" in this sense includes a repeating unit cell, preferably one that allows a 30 high density of beads on the substrate. However, it should be noted that these sites may not be discrete sites. That is, it is possible to use a uniform surface of adhesive or chemical functionalities, for example, that allows the attachment of beads at any position. That is, the surface of the substrate

is modified to allow attachment of the microspheres at individual sites, whether or not those sites are contiguous or non-contiguous with other sites. Thus, the surface of the substrate may be modified such that discrete sites are formed that can only have a single associated bead, or alternatively, the surface of the substrate is modified and beads may go down anywhere, but they end up at discrete sites.

In a preferred embodiment, the surface of the substrate is modified to contain wells, i.e. depressions in the surface of the substrate. This may be done as is generally known in the art using a variety of techniques, including, but not limited to, photolithography, stamping techniques, molding techniques and microetching techniques. As will be appreciated by those in the art, the technique used will depend on the composition and shape of the substrate.

In a preferred embodiment, physical alterations are made in a surface of the substrate to produce the sites. In a preferred embodiment, the substrate is a fiber optic bundle and the surface of the substrate is a terminal end of the fiber bundle. In this embodiment, wells are made in a terminal or distal end of a fiber optic bundle comprising individual fibers. In this embodiment, the cores of the individual fibers are etched, with respect to the cladding, such that small wells or depressions are formed at one end of the fibers. The required depth of the wells will depend on the size of the beads to be added to the wells.

Generally in this embodiment, the microspheres are non-covalently associated in the wells, although the wells may additionally be chemically functionalized as is generally described below, cross-linking agents may be used, or a physical barrier may be used, i.e. a film or membrane over the beads.

In a preferred embodiment, the surface of the substrate is modified to contain chemically modified sites, that can be used to attach, either covalently or non-covalently, the microspheres of the invention to the discrete sites or locations on the substrate. "Chemically modified sites" in this context includes, but is not limited to, the addition of a pattern of chemical functional groups including amino groups, carboxy groups, oxo groups and thiol groups, that can be used to covalently attach microspheres, which generally also contain corresponding reactive functional groups; the addition of a pattern of adhesive that can be used to bind the microspheres (either by prior chemical functionalization for the addition of the adhesive or direct addition of the adhesive); the addition of a pattern of charged groups (similar to the chemical functionalities) for the electrostatic attachment of the microspheres, i.e. when the microspheres comprise charged groups opposite to the sites; the addition of a pattern of chemical functional groups that renders the sites differentially hydrophobic or hydrophilic, such that the addition of similarly hydrophobic or hydrophilic microspheres under suitable experimental conditions will result in association of the microspheres to the sites on the basis of hydroaffinity. For example, the use of

5 hydrophobic sites with hydrophobic beads, in an aqueous system, drives the association of the beads preferentially onto the sites. As outlined above, "pattern" in this sense includes the use of a uniform treatment of the surface to allow attachment of the beads at discrete sites, as well as treatment of the surface resulting in discrete sites. As will be appreciated by those in the art, this may be accomplished in a variety of ways.

10 The compositions of the invention further comprise a population of microspheres. By "population" herein is meant a plurality of beads as outlined above for arrays. Within the population are separate subpopulations, which can be a single microsphere or multiple identical microspheres. That is, in some embodiments, as is more fully outlined below, the array may contain only a single bead for each bioactive agent; preferred embodiments utilize a plurality of beads of each type.

15 By "microspheres" or "beads" or "particles" or grammatical equivalents herein is meant small discrete particles. The composition of the beads will vary, depending on the class of bioactive agent and the method of synthesis. Suitable bead compositions include those used in peptide, nucleic acid and organic moiety synthesis, including, but not limited to, plastics, ceramics, glass, polystyrene, methylstyrene, acrylic polymers, paramagnetic materials, thoria sol, carbon graphited, titanium dioxide, latex or cross-linked dextrans such as Sepharose, cellulose, nylon, cross-linked micelles and teflon may all be used. *"Microsphere Detection Guide"* from Bangs Laboratories, Fishers IN is a helpful guide.

20 The beads need not be spherical; irregular particles may be used. In addition, the beads may be porous, thus increasing the surface area of the bead available for either bioactive agent attachment or tag attachment. The bead sizes range from nanometers, i.e. 100 nm, to millimeters, i.e. 1 mm, with beads from about 0.2 micron to about 200 microns being preferred, and from about 0.5 to about 5 micron being particularly preferred, although in some embodiments smaller beads may be used.

25 Fig. 1 illustrates the construction of a bead or microsphere 10 according to the principles of the present invention. In common with the prior art, the microsphere 10 is given a bioactive agent 12, which is typically applied to the microsphere's surface. The bioactive agent is designed so that in the presence of the analyte(s) to which it is targeted, an optical signature of the microsphere, possibly including region surrounding it, is changed.

30 It should be noted that a key component of the invention is the use of a substrate/bead pairing that allows the association or attachment of the beads at discrete sites on the surface of the substrate, such that the beads do not move during the course of the assay.

Each microsphere comprises two components: a bioactive agent and an optical signature.

By "candidate bioactive agent" or "bioactive agent" or "chemical functionality" or "binding ligand" herein is meant as used herein describes any molecule, e.g., protein, oligopeptide, small organic molecule, polysaccharide, polynucleotide, etc. which can be attached to the microspheres of the invention. It should be understood that the compositions of the invention have two primary uses. In a preferred embodiment, as is more fully outlined below, the compositions are used to detect the presence of a particular target analyte; for example, the presence or absence of a particular nucleotide sequence or a particular protein, such as an enzyme, an antibody or an antigen. In an alternate preferred embodiment, the compositions are used to screen bioactive agents, i.e. drug candidates, for binding to a particular target analyte.

Bioactive agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 100 and less than about 2,500 daltons. Bioactive agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The bioactive agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Bioactive agents are also found among biomolecules including peptides, nucleic acids, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Particularly preferred are nucleic acids and proteins.

Bioactive agents can be obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification and/or amidification to produce structural analogs.

In a preferred embodiment, the bioactive agents are proteins. By "protein" herein is meant at least two covalently attached amino acids, which includes proteins, polypeptides, oligopeptides and peptides. The protein may be made up of naturally occurring amino acids and peptide bonds, or synthetic peptidomimetic structures. Thus "amino acid", or "peptide residue", as used herein means both

5 naturally occurring and synthetic amino acids. For example, homo-phenylalanine, citrulline and norleucine are considered amino acids for the purposes of the invention. The side chains may be in either the (R) or the (S) configuration. In the preferred embodiment, the amino acids are in the (S) or L-configuration. If non-naturally occurring side chains are used, non-amino acid substituents may be used, for example to prevent or retard *in vivo* degradations.

10 In one preferred embodiment, the bioactive agents are naturally occurring proteins or fragments of naturally occurring proteins. Thus, for example, cellular extracts containing proteins, or random or directed digests of proteinaceous cellular extracts, may be used. In this way libraries of procaryotic and eukaryotic proteins may be made for screening in the systems described herein. Particularly preferred in this embodiment are libraries of bacterial, fungal, viral, and mammalian proteins, with the latter being preferred, and human proteins being especially preferred.

15 In a preferred embodiment, the bioactive agents are peptides of from about 5 to about 30 amino acids, with from about 5 to about 20 amino acids being preferred, and from about 7 to about 15 being particularly preferred. The peptides may be digests of naturally occurring proteins as is outlined above, random peptides, or "biased" random peptides. By "randomized" or grammatical equivalents herein is meant that each nucleic acid and peptide consists of essentially random nucleotides and amino acids, respectively. Since generally these random peptides (or nucleic acids, discussed below) are chemically synthesized, they may incorporate any nucleotide or amino acid at any position. The synthetic process can be designed to generate randomized proteins or nucleic acids, to allow the  
20 formation of all or most of the possible combinations over the length of the sequence, thus forming a library of randomized bioactive proteinaceous agents.

In a preferred embodiment, a library of bioactive agents are used. The library should provide a sufficiently structurally diverse population of bioactive agents to effect a probabilistically sufficient range of binding to target analytes. Accordingly, an interaction library must be large enough so that at  
25 least one of its members will have a structure that gives it affinity for the target analyte. Although it is difficult to gauge the required absolute size of an interaction library, nature provides a hint with the immune response: a diversity of  $10^7$ - $10^8$  different antibodies provides at least one combination with sufficient affinity to interact with most potential antigens faced by an organism. Published in vitro selection techniques have also shown that a library size of  $10^7$  to  $10^8$  is sufficient to find structures with  
30 affinity for the target. Thus, in a preferred embodiment, at least  $10^6$ , preferably at least  $10^7$ , more preferably at least  $10^8$  and most preferably at least  $10^9$  different bioactive agents are simultaneously analyzed in the subject methods. Preferred methods maximize library size and diversity.



In one embodiment, the library is fully randomized, with no sequence preferences or constants at any position. In a preferred embodiment, the library is biased. That is, some positions within the sequence are either held constant, or are selected from a limited number of possibilities. For example, in a preferred embodiment, the nucleotides or amino acid residues are randomized within a defined class, for example, of hydrophobic amino acids, hydrophilic residues, sterically biased (either small or large) residues, towards the creation of cysteines, for cross-linking, prolines for SH-3 domains, serines, threonines, tyrosines or histidines for phosphorylation sites, etc., or to purines, etc.

In a preferred embodiment, the bioactive agents are nucleic acids (generally called "probe nucleic acids" or "candidate probes" herein). By "nucleic acid" or "oligonucleotide" or grammatical equivalents hereir means at least two nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramidate (Beaucage, *et al.*, Tetrahedron, 49(10):1925 (1993) and references therein; Letsinger, J. Org. Chem., 35:3800 (1970); Sprinzl, *et al.*, Eur. J. Biochem., 81:579 (1977); Letsinger, *et al.*, Nucl. Acids Res., 14:3487 (1986); Sawai, *et al.*, Chem. Lett., 805 (1984); Letsinger, *et al.*, J. Am. Chem. Soc., 110:4470 (1988); and Pauwels, *et al.*, Chemica Scripta, 26:141 (1986)), phosphorothioate (Mag, *et al.*, Nucleic Acids Res., 19:1437 (1991); and U.S. Patent No. 5,644,048), phosphorodithioate (Briu, *et al.*, J. Am. Chem. Soc., 111:2321 (1989)), O-methylphosphoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, J. Am. Chem. Soc., 114:1895 (1992); Meier, *et al.*, Chem. Int. Ed. Engl., 31:1008 (1992); Nielsen, Nature, 365:566 (1993); Carlsson, *et al.*, Nature, 380:207 (1996), all of which are incorporated by reference)). Other analog nucleic acids include those with positive backbones (Denpcy, *et al.*, Proc. Natl. Acad. Sci. USA, 92:6097 (1995)); non-ionic backbones (U.S. Patent Nos. 5,386,023; 5,637,684; 5,602,240; 5,216,141; and 4,469,863; Kiedrowski, *et al.*, Angew. Chem. Intl. Ed. English, 30:423 (1991); Letsinger, *et al.*, J. Am. Chem. Soc., 110:4470 (1988); Letsinger, *et al.*, Nucleosides & Nucleotides, 13:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker, *et al.*, Bioorganic & Medicinal Chem. Lett., 4:395 (1994); Jeffs, *et al.*, J. Biomolecular NMR, 34:17 (1994); Tetrahedron Lett., 37:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins, *et al.*, Chem. Soc. Rev., (1995) pp. 169-176). Several nucleic acid analogs are described in Rawls, C & E News, June 2, 1997, page 35. All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of additional moieties such as labels, or to increase the stability and half-

life of such molecules in physiological environments. In addition, mixtures of naturally occurring nucleic acids and analogs can be made. Alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occurring nucleic acids and analogs may be made. The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid contains any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthanine, hypoxanthanine, isocytosine, isoguanine, and basepair analogs such as nitropyrrole and nitroindole, etc.

As described above generally for proteins, nucleic acid bioactive agents may be naturally occurring nucleic acids, random nucleic acids, or "biased" random nucleic acids. For example, digests of procaryotic or eukaryotic genomes may be used as is outlined above for proteins.

In general, probes of the present invention are designed to be complementary to a target sequence (either the target analyte sequence of the sample or to other probe sequences, as is described herein), such that hybridization of the target and the probes of the present invention occurs. This complementarity need not be perfect; there may be any number of base pair mismatches that will interfere with hybridization between the target sequence and the single stranded nucleic acids of the present invention. However, if the number of mutations is so great that no hybridization can occur under even the least stringent of hybridization conditions, the sequence is not a complementary target sequence. Thus, by "substantially complementary" herein is meant that the probes are sufficiently complementary to the target sequences to hybridize under the selected reaction conditions. High stringency conditions are known in the art; see for example Maniatis et al., *Molecular Cloning: A Laboratory Manual*, 2d Edition, 1989, and *Short Protocols in Molecular Biology*, ed. Ausubel, et al., both of which are hereby incorporated by reference. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point ( $T_m$ ) for the specific sequence at a defined ionic strength pH. The  $T_m$  is the temperature (under defined ionic strength, pH and nucleic acid concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at  $T_m$ , 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g. 10 to 50 nucleotides) and at least about 60°C for long probes (e.g. greater than 50 nucleotides). Stringent conditions may also

be achieved with the addition of destabilizing agents such as formamide. In another embodiment, less stringent hybridization conditions are used; for example, moderate or low stringency conditions may be used, as are known in the art; see Maniatis and Ausubel, *supra*, and Tijssen, *supra*.

5 The term "target sequence" or grammatical equivalents herein means a nucleic acid sequence on a single strand of nucleic acid. The target sequence may be a portion of a gene, a regulatory sequence, genomic DNA, cDNA, RNA including mRNA and rRNA, or others. It may be any length, with the understanding that longer sequences are more specific. As will be appreciated by those in the art, the complementary target sequence may take many forms. For example, it may be contained within a larger nucleic acid sequence, i.e. all or part of a gene or mRNA, a restriction fragment of a plasmid or  
10 genomic DNA, among others. As is outlined more fully below, probes are made to hybridize to target sequences to determine the presence or absence of the target sequence in a sample. Generally speaking, this term will be understood by those skilled in the art.

In a preferred embodiment, the bioactive agents are organic chemical moieties, a wide variety of which are available in the literature.

15 In a preferred embodiment, each bead comprises a single type of bioactive agent, although a plurality of individual bioactive agents are preferably attached to each bead. Similarly, preferred embodiments utilize more than one microsphere containing a unique bioactive agent; that is, there is redundancy built into the system by the use of subpopulations of microspheres, each microsphere in the subpopulation containing the same bioactive agent.

20 As will be appreciated by those in the art, the bioactive agents may either be synthesized directly on the beads, or they may be made and then attached after synthesis. In a preferred embodiment, linkers are used to attach the bioactive agents to the beads, to allow both good attachment, sufficient flexibility to allow good interaction with the target molecule, and to avoid undesirable binding reactions.

25 In a preferred embodiment, the bioactive agents are synthesized directly on the beads. As is known in the art, many classes of chemical compounds are currently synthesized on solid supports, such as peptides, organic moieties, and nucleic acids. It is a relatively straightforward matter to adjust the current synthetic techniques to use beads.

30 In a preferred embodiment, the bioactive agents are synthesized first, and then covalently attached to the beads. As will be appreciated by those in the art, this will be done depending on the composition of the bioactive agents and the beads. The functionalization of solid support surfaces such as certain

polymers with chemically reactive groups such as thiols, amines, carboxyls, etc. is generally known in the art. Accordingly, "blank" microspheres may be used that have surface chemistries that facilitate the attachment of the desired functionality by the user. Some examples of these surface chemistries for blank microspheres are listed in Table I.

Table I

<u>Surface chemistry</u>	<u>Name:</u>
NH <sub>2</sub>	Amine
COOH	Carboxylic Acid
CHO	Aldehyde
CH <sub>2</sub> -NH <sub>2</sub>	Aliphatic Amine
CO NH <sub>2</sub>	Amide
CH <sub>2</sub> -Cl	Chloromethyl
CONH-NH <sub>2</sub>	Hydrazide
OH	Hydroxyl
SO <sub>4</sub>	Sulfate
SO <sub>3</sub>	Sulfonate
Ar NH <sub>2</sub>	Aromatic Amine

These functional groups can be used to add any number of different bioactive agents to the beads, generally using known chemistries. For example, bioactive agents containing carbohydrates may be attached to an amino-functionalized support; the aldehyde of the carbohydrate is made using standard techniques, and then the aldehyde is reacted with an amino group on the surface. In an alternative embodiment, a sulfhydryl linker may be used. There are a number of sulfhydryl reactive linkers known in the art such as SPDP, maleimides,  $\alpha$ -haloacetyls, and pyridyl disulfides (see for example the 1994 Pierce Chemical Company catalog, technical section on cross-linkers, pages 155-200, incorporated herein by reference) which can be used to attach cysteine containing proteinaceous agents to the support. Alternatively, an amino group on the bioactive agent may be used for attachment to an amino group on the surface. For example, a large number of stable bifunctional groups are well known in the art, including homobifunctional and heterobifunctional linkers (see Pierce Catalog and Handbook, pages 155-200). In an additional embodiment, carboxyl groups (either from the surface or from the bioactive agent) may be derivatized using well known linkers (see the Pierce catalog). For example, carbodiimides activate carboxyl groups for attack by good nucleophiles such as amines (see Torchilin et al., Critical Rev. Therapeutic Drug Carrier Systems, 7(4):275-308 (1991), expressly incorporated herein). Proteinaceous bioactive agents may also be attached using other techniques known in the

art, for example for the attachment of antibodies to polymers; see Slinkin et al., Bioconj. Chem. 2:342-348 (1991); Torchilin et al., *supra*; Trubetskoy et al., Bioconj. Chem. 3:323-327 (1992); King et al., Cancer Res. 54:6176-6185 (1994); and Wilbur et al., Bioconjugate Chem. 5:220-235 (1994), all of which are hereby expressly incorporated by reference). It should be understood that the bioactive agents may be attached in a variety of ways, including those listed above. What is important is that the manner of attachment does not significantly alter the functionality of the bioactive agent; that is, the bioactive agent should be attached in such a flexible manner as to allow its interaction with a target.

Specific techniques for immobilizing enzymes on microspheres are known in the prior art. In one case,  $\text{NH}_2$  surface chemistry microspheres are used. Surface activation is achieved with a 2.5% glutaraldehyde in phosphate buffered saline (10 mM) providing a pH of 6.9. (138 mM NaCl, 2.7 mM, KCl). This is stirred on a stir bed for approximately 2 hours at room temperature. The microspheres are then rinsed with ultrapure water plus 0.01% tween 20 (surfactant) -0.02%, and rinsed again with a pH 7.7 PBS plus 0.01% tween 20. Finally, the enzyme is added to the solution, preferably after being prefiltered using a 0.45 $\mu\text{m}$  amicon micropure filter.

In addition to a bioactive agent, the microspheres comprise an optical signature that can be used to identify the attached bioactive agent. That is, each subpopulation of microspheres comprise a unique optical signature or optical tag that can be used to identify the unique bioactive agent of that subpopulation of microspheres; a bead comprising the unique optical signature may be distinguished from beads at other locations with different optical signatures. As is outlined herein, each bioactive agent will have an associated unique optical signature such that any microspheres comprising that bioactive agent will be identifiable on the basis of the signature. As is more fully outlined below, it is possible to reuse or duplicate optical signatures within an array, for example, when another level of identification is used, for example when beads of different sizes are used, or when the array is loaded sequentially with different batches of beads.

In a preferred embodiment, the optical signature is generally a mixture of reporter dyes, preferably fluorescent. By varying both the composition of the mixture (i.e. the ratio of one dye to another) and the concentration of the dye (leading to differences in signal intensity), matrices of unique tags may be generated. This may be done by covalently attaching the dyes to the surface of the beads, or alternatively, by entrapping the dye within the bead. The dyes may be chromophores or phosphors but are preferably fluorescent dyes, which due to their strong signals provide a good signal-to-noise ratio for decoding. Suitable dyes for use in the invention include, but are not limited to, fluorescent lanthanide complexes, including those of Europium and Terbium, fluorescein, rhodamine, tetramethylrhodamine, eosin, erythrosin, coumarin, methyl-coumarins, pyrene, Malacite green,

stilbene, Lucifer Yellow, Cascade Blue™, Texas Red, and others described in the 1989-1991 Molecular Probes Handbook by Richard P. Haugland, hereby expressly incorporated by reference.

5 In a preferred embodiment, the encoding can be accomplished in a ratio of at least two dyes, although more encoding dimensions may be added in the size of the beads, for example. In addition, the labels are distinguishable from one another; thus two different labels may comprise different molecules (i.e. two different fluors) or, alternatively, one label at two different concentrations or intensity.

10 In a preferred embodiment, the dyes are covalently attached to the surface of the beads. This may be done as is generally outlined for the attachment of the bioactive agents, using functional groups on the surface of the beads. As will be appreciated by those in the art, these attachments are done to minimize the effect on the dye.

15 In a preferred embodiment, the dyes are non-covalently associated with the beads, generally by entrapping the dyes in the bead matrix or pores of the beads. Referring to the embodiment of Figure 1, reporter dyes 14 are added to the microsphere 10 with the encoding occurring in the ratio of two or more dyes. The reporter dyes 14 may be chromophore-type. Fluorescent dyes, however, are preferred because the strength of the fluorescent signal provides a better signal-to-noise ratio when decoding. Additionally, encoding in the ratios of the two or more dyes, rather than single dye concentrations, is preferred since it provides insensitivity to the intensity of light used to interrogate the reporter dye's signature and detector sensitivity.

20 In one embodiment, the dyes are added to the bioactive agent, rather than the beads, although this is generally not preferred.

25 Fig. 2 is a process diagram illustrating the preparation of the microspheres. In step 50, an aliquot of stock microspheres are vacuum filtered to produce a dry cake. In one implementation, microsphere copolymers of methylstyrene (87%) and divinylbenzene (13%) are used that have a 3.1 micrometer (μm) diameter. The dry cake is then broken apart and a dye solution added to it in step 52 to encode optical signatures of the microspheres with information concerning the intended surface chemical functionalities. Dyes may be covalently bonded to the microspheres' surface, but this consumes surface binding sites desirably reserved for the chemical functionalities. Preferably, the microspheres are placed in a dye solution comprising a ratio of two or more fluorescent reporter dyes dissolved in an organic solvent that will swell the microspheres, e.g., dimethylformamide (DMF). The length of time the  
30 microspheres are soaked in the dye solution will determine their intensity and the broadness of the ratio range.

In an exemplary two dye system, Texas Red Cadaverine (TRC) is used, which is excited at  $\lambda_{ab}=580$  nm and emits at  $\lambda_{em}=630$  nm, in combination with indodicarbocyanine (DiIC): 610/670 ( $\lambda_{ab}/\lambda_{em}$ ). Generally, dyes are selected to be compatible with the chemistries involved in the analysis and to be spectrally compatible. This avoids deconvolution problems associated with determining signal contributions based on the presence of both the analyte and the encoding dye ratios contributing to an overlapping emission spectral region.

Examples of other dyes that can be used are Oxazin (662/705), IR-144 (745/825), IR-140 (776/882), IR-125 (786/800) from Exciton, and Bodipy 665/676 from Molecular Probes, and Naphthofluorescein (605/675) also from Molecular Probes. Lanthanide complexes may also be used. Fluorescent dyes emitting in other than the near infrared may also be used. Chromophore dyes are still another alternative that produce an optically interrogatable signature, as are more exotic formulations using Raman scattering-based dyes or polarizing dyes, for example. The ability of a particular dye pair to encode for different chemical functionalities depends on the resolution of the ratiometric measurement. Conservatively, any dye pair should provide the ability to discriminate at least twenty different ratios. The number of unique combinations of two dyes made with a particular dye set is shown in the following Table II.

Table II

Number of dyes in set	Combinations possible
3	3
4	6
5	10
6	15

Thus, using six dyes and twenty distinct ratios for each dye pair, 300 separate chemical functionalities may be encoded in a given population of microspheres. Combining more than two dyes provides additional diversity in the encoding combinations. Furthermore, the concentration of the dyes will contribute to their intensity; thus intensity is another way to increase the number of unique optical signatures.

In step 54, the microspheres are vacuum filtered to remove excess dye. The microspheres are then washed in water or other liquid that does not swell the microspheres, but in which the dyes are still soluble. This allows the residual dye to be rinsed off without rinsing the dye out of the microspheres.

In step 56, the bioactive agent is attached to the microsphere surface if not already present. It should be understood that surface chemistries may be present throughout the microsphere's volume, and not limited to the physical circumferential surface.

5 Once the microspheres are made comprising at least one bioactive agent and an optical signature, the microspheres are added to discrete sites on the surface of the substrate. This can be done in a number of ways, but generally comprises adding the beads to the surface under conditions that will allow the association of the microspheres on or at the discrete sites. The association of the beads on the surface may comprise a covalent bonding of the bead to the surface, for example when chemical attachment sites are added to both the substrate and the bead; an electrostatic or hydroaffinity, when charge, hydrophobicity or hydrophilicity is used as the basis of the binding; a physical yet non-covalent attachment such as the use of an adhesive; or a spatial attachment, for example the localization of a bead within a well. In some embodiments it may be preferable to effect a more permanent attachment after the initial localization, for example through the use of cross-linking agents, a film or membrane over the array.

15 Fig. 3 schematically illustrates a microsphere system, or array of microspheres, 100 formed from microsphere populations that have different bioactive agents. While a large number of microspheres and bioactive agents may be employed, in this example only three microsphere populations are shown. The individual populations, or subpopulations, of microspheres are represented as IOa, IOb, IOc carrying respective bioactive agents or probe sequences 60a, 60b, 60c, as exemplary  
20 functionalities. The subpopulations may be combined in either a random or ordered fashion on a substrate, with a corresponding distribution of their respective bioactive agents.

Typically, with conventional methods, mixing microsphere populations having different bioactive agents results in the loss of information regarding the selectivity for each of the corresponding target sequences. In a solution of microspheres with each of the probe sequences 60a, 60b, and 60c, it is  
25 possible to determine that at least one of the target sequences 62a, 62b, and 62c is present when a fluorescent marker dye 64 concentration is observed on the microspheres 10. However, with conventional approaches, typically there is no way to determine which bioactive agent or probe sequence 60a, 60b, and 60c is generating the activity since the information concerning which microsphere contained which probe sequence was lost when the subpopulations were mixed.

30 However, with the microsphere system 100 and method of the present invention, each microsphere in each subpopulation is encoded with a common optical signature. In the illustrated example, the subpopulation represented by microsphere 10a has a two reporter dye ratio of 10:1; the subpopulation



of microspheres 10b has a ratio of 1:1 of the same reporter dyes, and subpopulation of microspheres 10c has a ratio of 1:10 of the reporter dyes.

Thus, the randomly mixed subpopulations of microspheres are useful as an analytic chemistry system based on each of the carried bioactive agents 60a-60c separately. The microsphere array or system 100 is exposed to an analyte of interest to which some of the bioactive agents may interact. Any interaction changes the optical response of the corresponding microspheres by, for example, binding a fluorescent dye 64 to the microspheres. By identifying the chemical functionalities of the microsphere in which the optical signature has changed, using the encoded dye combinations, information regarding the chemical identity and concentration of an analyte may be gained based upon the interaction or noninteraction of each bioactive agent contained in the microsphere system 100.

The microspheres exhibiting activity or changes in their optical signature may be identified by a conventional optical train and optical detection system. Decoding can also be performed either manually or automatically with the aid of a computer. Depending on the particular encoding or reporter dyes used and their operative wavelengths, optical filters designed for a particular wavelengths may be employed for optical interrogation of the microspheres of bioactive agents. In a preferred embodiment, the analytic chemistry microsphere system is used in conjunction with an optical fiber bundle or fiber optic array as a substrate.

Fig. 4 is a schematic block diagram showing a microsphere-based analytic chemistry system employing a fiber optic assembly 200 with an optical detection system. The fiber optic assembly 200 comprises a fiber optic bundle or array 202, that is constructed from clad fibers so that light does not mix between fibers. A microsphere array or system, 100 is attached to the bundle's distal end 212, with the proximal end 214 being received by a z-translation stage 216 and x-y micropositioner 218. These two components act in concert to properly position the proximal end 214 of the bundle 202 for a microscope objective lens 220. Light collected by the objective lens 220 is passed to a reflected light fluorescence attachment with three pointer cube slider 222. The attachment 222 allows insertion of light from a 75 Watt Xe lamp 224 through the objective lens 220 to be coupled into the fiber bundle 202. The light from the source 224 is condensed by condensing lens 226, then filtered and/or shuttered by filter and shutter wheel 228, and subsequently passes through a ND filter slide 230.

Light returning from the distal end 212 of the bundle 202 is passed by the attachment 222 to a magnification changer 232 which enables adjustment of the image size of the fiber's proximal or distal end. Light passing through the magnification changer 232 is then shuttered and filtered by a second wheel 234. The light is then imaged on a charge coupled device (CCD) camera 236. A computer 238 executes imaging processing software to process the information from the CCD camera 236 and also

possibly control the first and second shutter and filter wheels 228, 234. The instrumentation exclusive of the fiber sensor 200, *i.e.*, to the left of the proximal end of the bundle 202 is discussed more completely by Bronk, et al., Anal. Chem. 1995, Vol. 67, number 17, pp. 2750-2752.

5 The microsphere array or system 100 may be attached to the distal end of the optical fiber bundle using a variety of compatible processes. It is important that the microspheres are located close to the end of the bundle. This ensures that the light returning in each optical fiber predominantly comes from only a single microsphere. This feature is necessary to enable the interrogation of the optical signature of individual microspheres to identify reactions involving the microsphere's functionality and also to  
10 decode the dye ratios contained in those microspheres. The adhesion or affixing technique, however, must not chemically insulate the microspheres from the analyte.

Figs. 5A and 5B are micrographs of the distal end 212 of the bundle 202 illustrating the preferred technique for attaching the microspheres 10 to the bundle 202. Wells 250 are formed at the center of each optical fiber 252 of the bundle 202. As shown in Fig. 5B, the size of the wells 250 are coordinated with the size of the microspheres 10 so that the microspheres 10 can be placed within the wells 250.  
15 Thus, each optical fiber 252 of the bundle 202 conveys light from the single microsphere 10 contained in its well. Consequently, by imaging the end of the bundle 202 onto the CCD array 236, the optical signatures of the microspheres 10 are individually interrogatable.

Fig. 6 illustrates how the microwells 250 are formed and microspheres 10 placed in the wells. A 1 mm hexagonally-packed imaging fiber contains approximately 20,600 individual optical fibers that have  
20 cores approximately 3.7 $\mu$ m across (Part No. ET26 from Galileo Fibers). Typically, the cores of each fiber are hexagonally shaped as a result of the starting preform; that is, during drawing the fiber does not usually change shape. In some cases, the shape can be circular, however.

In step 270, both the proximal and distal ends 212, 214 of the fiber bundle 202 are successively polished on 12  $\mu$ m, 9  $\mu$ m, 3  $\mu$ m, 1  $\mu$ m, and 0.3  $\mu$ m lapping films. Subsequently, the ends can be  
25 inspected for scratches on an atomic force microscope. In step 272, a representative etching is performed on the distal end 212 of the bundle 202. A solution of 0.2 grams  $\text{NH}_4\text{F}$  (ammonium fluoride) with 600  $\mu$ l distilled  $\text{H}_2\text{O}$  and 100  $\mu$ l of HF (hydrofluoric acid), 50% stock solution, may be used. The distal end 212 is etched in this solution for a specified time, preferably approximately 30 to 600 seconds, with about 80 seconds being preferred.

30 Upon removal from this solution, the bundle end is immediately placed in deionized water to stop any further etching in step 274. The fiber is then rinsed in running tap water. At this stage, sonication is

preferably performed for several minutes to remove any salt products from the reaction. The fiber is then allowed to air dry.

5 The foregoing procedure produces wells by the anisotropic etching of the fiber cores 254 favorably with respect to the cladding 256 for each fiber of the bundle 202. The wells have approximately the diameter of the cores 254, 3.7  $\mu\text{m}$ . This diameter is selected to be slightly larger than the diameters of the microspheres used, 3.1  $\mu\text{m}$ , in the example. The preferential etching occurs because the pure silica of the cores 254 etches faster in the presence of hydrofluoric acid than the germanium-doped silica claddings 256.

10 The microspheres are then placed in the wells 250 in step 276 according to a number of different techniques. The placement of the microspheres may be accomplished by dripping a solution containing the desired randomly mixed subpopulations of the microspheres over the distal end 212, sonicating the bundle to settle the microspheres in the wells, and allowing the microsphere solvent to evaporate. Alternatively, the subpopulations could be added serially to the bundle end. Microspheres 10 may then be fixed into the wells 250 by using a dilute solution of sulfonated Nafion that is dripped 15 over the end. Upon solvent evaporation, a thin film of Nafion was formed over the microspheres which holds them in place. This approach is compatible for fixing microspheres for pH indication that carry FITC functionality. The resulting array of fixed microspheres retains its pH sensitivity due to the permeability of the sulfonated Nafion to hydrogen ions. This approach, however, can not be employed generically as Nafion is impermeable to most water soluble species. A similar approach can be 20 employed with different polymers. For example, solutions of polyethylene glycol, polyacrylamide, or polyhydroxymethyl methacrylate (polyHEMA) can be used in place of Nafion, providing the requisite permeability to aqueous species.

25 An alternative fixation approach employs microsphere swelling to entrap each microsphere 10 in its corresponding microwell 250. In this approach, the microspheres are first distributed into the microwells 250 by sonicating the microspheres suspended in a non-swelling solvent in the presence of the microwell array on the distal end 212. After placement into the microwells, the microspheres are subsequently exposed to an aqueous buffer in which they swell, thereby physically entrapping them, analogous to muffins rising in a muffin tin.

30 In general, the methods of making the arrays and of decoding the arrays is done to maximize the number of different candidate agents that can be uniquely encoded. The compositions of the invention may be made in a variety of ways. In general, the arrays are made by adding a solution or slurry comprising the beads to a surface containing the sites for attachment of the beads. This may be done

in a variety of buffers, including aqueous and organic solvents, and mixtures. The solvent can evaporate, and excess beads removed.

5 In a preferred embodiment, when non-covalent methods are used to associate the beads to the array, a novel method of loading the beads onto the array is used. This method comprises exposing the array to a solution of particles (including microspheres and cells) and then applying energy, e.g. agitating or vibrating the mixture. This results in an array comprising more tightly associated particles, as the agitation is done with sufficient energy to cause weakly-associated beads to fall off (or out, in the case of wells). These sites are then available to bind a different bead. In this way, beads that exhibit a high affinity for the sites are selected. Arrays made in this way have two main advantages as compared to a more static loading: first of all, a higher percentage of the sites can be filled easily, and secondly, the arrays thus loaded show a substantial decrease in bead loss during assays. Thus, in a preferred embodiment, these methods are used to generate arrays that have at least about 50% of the sites filled, with at least about 75% being preferred, and at least about 90% being particularly preferred. Similarly, arrays generated in this manner preferably lose less than about 20% of the beads during an assay, with less than about 10% being preferred and less than about 5% being particularly preferred.

20 In this embodiment, the substrate comprising the surface with the discrete sites is immersed into a solution comprising the particles (beads, cells, etc.). The surface may comprise wells, as is described herein, or other types of sites on a patterned surface such that there is a differential affinity for the sites. This differential affinity results in a competitive process, such that particles that will associate more tightly are selected. Preferably, the entire surface to be "loaded" with beads is in fluid contact with the solution. This solution is generally a slurry ranging from about 10,000:1 beads:solution (vol:vol) to 1:1. Generally, the solution can comprise any number of reagents, including aqueous buffers, organic solvents, salts, other reagent components, etc. In addition, the solution preferably comprises an excess of beads; that is, there are more beads than sites on the array. Preferred embodiments utilize two-fold to billion-fold excess of beads.

The immersion can mimic the assay conditions; for example, if the array is to be "dipped" from above into a microtiter plate comprising samples, this configuration can be repeated for the loading, thus minimizing the beads that are likely to fall out due to gravity.

30 Once the surface has been immersed, the substrate, the solution, or both are subjected to a competitive process, whereby the particles with lower affinity can be disassociated from the substrate and replaced by particles exhibiting a higher affinity to the site. This competitive process is done by

the introduction of energy, in the form of heat, sonication, stirring or mixing, vibrating or agitating the solution or substrate, or both.

5 A preferred embodiment utilizes agitation or vibration. In general, the amount of manipulation of the substrate is minimized to prevent damage to the array; thus, preferred embodiments utilize the agitation of the solution rather than the array, although either will work. As will be appreciated by those in the art, this agitation can take on any number of forms, with a preferred embodiment utilizing microtiter plates comprising bead solutions being agitated using microtiter plate shakers.

10 The agitation proceeds for a period of time sufficient to load the array to a desired fill. Depending on the size and concentration of the beads and the size of the array, this time may range from about 1 second to days, with from about 1 minute to about 24 hours being preferred.

It should be noted that not all sites of an array may comprise a bead; that is, there may be some sites on the substrate surface which are empty. In addition, there may be some sites that contain more than one bead, although this is not preferred.

15 One of the most common microsphere formations is tentagel, a styrene-polyethylene glycol co-polymer. These microspheres are unswollen in nonpolar solvents such as hexane and swell approximately 20-40% in volume upon exposure to a more polar or aqueous media. This approach is extremely desirable since it does not significantly compromise the diffusional or permeability properties of the microspheres themselves.

20 Figs. 7A and 7B show polymer coated microspheres 12 in wells 250 after their initial placement and then after tapping and exposure to air pulses. Figs. 7A and 7B illustrate that there is no appreciable loss of microspheres from the wells due to mechanical agitation even without a specific fixing technique. This effect is probably due to electrostatic forces between the microspheres and the optical fibers. These forces tend to bind the microspheres within the wells. Thus, in most environments, it may be unnecessary to use any chemical or mechanical fixation for the microspheres.

25 In a preferred embodiment, particularly when wells are used, a sonication step may be used to place beads in the wells.

It should be noted that not all sites of an array may comprise a bead; that is, there may be some sites on the substrate surface which are empty. In addition, there may be some sites that contain more than one bead, although this is not preferred.

In some embodiments, for example when chemical attachment is done, it is possible to attach the beads in a non-random or ordered way. For example, using photoactivatable attachment linkers or photoactivatable adhesives or masks, selected sites on the array may be sequentially rendered suitable for attachment, such that defined populations of beads are laid down.

5 In addition, since the size of the array will be set by the number of unique optical signatures, it is possible to "reuse" a set of unique optical signatures to allow for a greater number of test sites. This may be done in several ways; for example, by using a positional coding scheme within an array; different sub-bundles may reuse the set of optical signatures. Similarly, one embodiment utilizes bead size as a coding modality, thus allowing the reuse of the set of unique optical signatures for each bead  
10 size. Alternatively, sequential partial loading of arrays with beads can also allow the reuse of optical signatures.

In a preferred embodiment, a spatial or positional coding system is done. In this embodiment, there are sub-bundles or subarrays (i.e. portions of the total array) that are utilized. By analogy with the telephone system, each subarray is an "area code", that can have the same tags (i.e. telephone  
15 numbers) of other subarrays, that are separated by virtue of the location of the subarray. Thus, for example, the same unique tags can be reused from bundle to bundle. Thus, the use of 50 unique tags in combination with 100 different subarrays can form an array of 5000 different bioactive agents. In this embodiment, it becomes important to be able to identify one bundle from another; in general, this is done either manually or through the use of marker beads, i.e. beads containing unique tags for each  
20 subarray.

In alternative embodiments, additional encoding parameters can be added, such as microsphere size. For example, the use of different size beads may also allow the reuse of sets of optical signatures; that is, it is possible to use microspheres of different sizes to expand the encoding dimensions of the microspheres. Optical fiber arrays can be fabricated containing pixels with different fiber diameters or  
25 cross-sections; alternatively, two or more fiber optic bundles, each with different cross-sections of the individual fibers, can be added together to form a larger bundle; or, fiber optic bundles with fiber of the same size cross-sections can be used, but just with different sized beads. With different diameters, the largest wells can be filled with the largest microspheres and then moving onto progressively smaller microspheres in the smaller wells until all size wells are then filled. In this manner, the same dye ratio could be used to encode microspheres of different sizes thereby expanding the number of different  
30 oligonucleotide sequences or chemical functionalities present in the array. Although outlined for fiber optic substrates, this as well as the other methods outlined herein can be used with other substrates and with other attachment modalities as well.

In a preferred embodiment, the coding and decoding is accomplished by sequential loading of the microspheres into the array. As outlined above for spatial coding, in this embodiment, the optical signatures can be "reused". In this embodiment, the library of microspheres each comprising a different bioactive agent (or the subpopulations each comprise a different bioactive agent), is divided into a plurality of sublibraries; for example, depending on the size of the desired array and the number of unique tags, 10 sublibraries each comprising roughly 10% of the total library may be made, with each sublibrary comprising roughly the same unique tags. Then, the first sublibrary is added to the fiber optic bundle comprising the wells, and the location of each bioactive agent is determined, using its optical signature. The second sublibrary is then added, and the location of each optical signature is again determined. The signal in this case will comprise the "first" optical signature and the "second" optical signature; by comparing the two matrices the location of each bead in each sublibrary can be determined. Similarly, adding the third, fourth, etc. sublibraries sequentially will allow the array to be filled.

Thus, arrays are made of a large spectrum of chemical functionalities utilizing the compositions of invention comprising microspheres and substrates with discrete sites on a surface. Specifically, prior art sensors which can be adapted for use in the present invention include four broad classifications of microsphere sensors: 1) basic indicator chemistry sensors; 2) enzyme-based sensors; 3) immuno-based sensors (both of which are part of a broader general class of protein sensors); and 4) geno-sensors.

In a preferred embodiment, the bioactive agents are used to detect chemical compounds. A large number of basic indicator sensors have been previously demonstrated. Examples include:

Table III		
TARGET ANALYTE	Bioactive agent	NOTES ( $\lambda_{AB}/\lambda_{EM}$ )
pH Sensors based on:	seminaphthofluoresceins	e.g., carboxyl-SNAFL
	seminaphthorhodafluors	e.g., carboxyl-SNARF
	8-hydroxypyrene-1,3,6-trisulfonic acid	
	fluorescein	
CO <sub>2</sub> Sensors based On:	seminaphthofluoresceins	e.g., carboxyl-SNAFL
	seminaphthorhodafluors	e.g., carboxyl-SNARF
	8-hydroxypyrene-1,3,6-trisulfonic acid	

Table III		
Metal Ions Sensors based on:	desferriozamine B	e.g., Fe
	cyclen derivative	e.g., Cu, Zn
	derivatized peptides	e.g., FITC-Gly-Gly-His, and FITC-Gly His, Cu, Zn
	fluorexon (calcein)	e.g., Ca, Mg, Cu, Pb, Ba
	calcein blue	e.g., Ca, Mg, Cu
	methyl calcein blue	e.g., Ca, Mg, Cu
	ortho-dianisidine tetracetic acid (ODTA)	e.g., Zn
	bis-salicylidene ethylenediamine (SED)	e.g., Al
	N-(6-methoxy-8-quinolyl-p- toluenesulfonamide (TSQ)	e.g., Zn
	Indo-1	e.g., Mn, Ni
	Fura-2	e.g., Mn, Ni
	Magesium Green	e.g., Mg, Cd, Tb
O <sub>2</sub>	Siphenylisobenzofuran	409/476
	Methoxyvinyl pyrene	352/401
Nitrite	diaminonaphthalene	340/377
NO	luminol	355/411
	dihydrohodamine	289/none
Ca <sup>2+</sup>	Bis-fura	340/380
	Calcium Green	visible light/530
	Fura-2	340/380
	Indo-1	405/485
	Fluo-3	visible light/525
	Rhod-2	visible light/570
Mg <sup>2+</sup>	Mag-Fura-2	340/380
	Mag-Fura-5	340/380
	Mag-Indo-1	405/485
	Magnesium Green	475/530



Table III		
	Magnesium Orange	visible light/545
Zn <sup>2+</sup>	Newport Green	506/535
TSQ	Methoxy-Quinobyl	334/385
Cu <sup>+</sup>	Phen Green	492/517
Na <sup>+</sup>	SBFI	339/565
	SBFO	354/575
	Sodium Green	506/535
K <sup>+</sup>	PBFI	336/557
Cl <sup>-</sup>	SPQ	344/443
	MQAE	350/460

Each of the chemicals listed in Table III directly produces an optically interrogatable signal or a change in the optical signature, as is more fully outlined below, in the presence of the targeted analyte.

Enzyme-based microsphere sensors have also been demonstrated and could be manifest on microspheres. Examples include:

Table IV

SENSOR TARGET	Bioactive agent
Glucose Sensor	glucose oxidase (enz.) + O <sub>2</sub> -sensitive dye (see Table I)
Penicillin Sensor	penicillinase (enz.) + pH-sensitive dye (see Table I)
Urea Sensor	urease (enz.) + pH-sensitive dye (see Table I)
Acetylcholine Sensor	acetylcholinesterase (enz.) + pH-sensitive dye (see Table I)

Generally, as more fully outlined above, the induced change in the optical signal due to the presence of the enzyme-sensitive chemical analyte occurs indirectly in this class of chemical functionalities. The microsphere-bound enzyme, e.g., glucose oxidase, decomposes the target analyte, e.g., glucose, consume a co-substrate, e.g., oxygen, or produce some by-product, e.g., hydrogen peroxide. An oxygen sensitive dye is then used to trigger the signal change.

Immuno-based microsphere sensors have been demonstrated for the detection for environmental pollutants such as pesticides, herbicides, PCB's and PAH's. Additionally, these sensors have also been used for diagnostics, such as bacterial (e.g., leprosy, cholera, lyme disease, and tuberculosis), viral (e.g., HIV, herpes simplex, cytomegalovirus), fungal (e.g., aspergillosis, candidiasis, cryptococcoses), Mycoplasmal (e.g., mycoplasmal pneumonia), Protozoal (e.g., amoebiasis, toxoplasmosis), Rickettsial (e.g., Rocky Mountain spotted fever), and pregnancy tests.

Microsphere genosensors may also be made (see the Examples). These are typically constructed by attaching a probe sequence to the microsphere surface chemistry, typically via an  $\text{NH}_2$  group. A fluorescent dye molecule, e.g., fluorescein, is attached to the target sequence, which is in solution. The optically interrogatable signal change occurs with the binding of the target sequences to the microsphere. This produces a higher concentration of dye surrounding the microsphere than in the solution generally. A few demonstrated probe and target sequences, see Ferguson, J.A. et al. *Nature Biotechnology*, Vol. 14, Dec. 1996, are listed below in Table V.

Table V

PROBE SEQUENCES	TARGET SEQUENCES
B-glo(+) (segment of human B-globin)5'-NH <sub>2</sub> -(CH <sub>2</sub> ) <sub>8</sub> -TT TTT TTT TCA ACT TCA TCC ACG TTC ACC-3'	B-glo(+)-CF 5'-Fluorescein-TC AAC GTG GAT GAA GTT C-3'
IFNG(interferon gamma 1)5'-NH <sub>2</sub> -(CH <sub>2</sub> ) <sub>8</sub> -T <sub>12</sub> -TGG CTT CTC TTG GCT GTT ACT-3'	IFNG-CF 5'-Fluorescein-AG TAA CAG CCA AGA GAA CCC AAA-3'
IL2(interleukin-2)5'-NH <sub>2</sub> -(CH <sub>2</sub> ) <sub>8</sub> -T <sub>12</sub> -TA ACC GAA TCC CAA ACT CAC CAG-3'	IL2-CF 5'-Fluorescein-CT GGT GAG TTT GGG ATT CTT GTA-3'
IL4(interleukin-4)5'-NH <sub>2</sub> -(CH <sub>2</sub> ) <sub>8</sub> -T <sub>12</sub> -CC AAC TGC TTC CCC CTC TGT-3'	IL4-CF 5'-Fluorescein-AC AGA GGG GGA AGC AGT TGG-3'
IL6(interleukin-6)5'-NH <sub>2</sub> -(CH <sub>2</sub> ) <sub>8</sub> -T <sub>12</sub> -GT TGG GTC AGG GGT GGT TAT T-3'	IL6-CF 5'-Fluorescein-AA TAA CCA CCC CTG ACC CAA C-3'

It should be further noted that the genosensors can be based on the use of hybridization indicators as the labels. Hybridization indicators preferentially associate with double stranded nucleic acid, usually reversibly. Hybridization indicators include intercalators and minor and/or major groove binding moieties. In a preferred embodiment, intercalators may be used; since intercalation generally only

occurs in the presence of double stranded nucleic acid, only in the presence of target hybridization will the label light up.

5 The present invention may be used with any or all of these types of sensors. As will be appreciated by those in the art, the type and composition of the sensor will vary widely, depending on the composition of the target analyte. That is, sensors may be made to detect nucleic acids, proteins (including enzyme sensors and immunosensors), lipids, carbohydrates, etc; similarly, these sensors may include bioactive agents that are nucleic acids, proteins, lipids, carbohydrates, etc. In addition, a single array sensor may contain different binding ligands for multiple types of analytes; for example, an array sensor for HIV may contain multiple nucleic acid probes for direct detection of the viral genome, protein binding ligands for direct detection of the viral particle, immuno-components for the detection of anti-HIV antibodies, etc.

10 In addition to the beads and the substrate, the compositions of the invention may include other components, such as light sources, optical components such as lenses and filters, detectors, computer components for data analysis, etc.

15 The arrays of the present invention are constructed such that information about the identity of the bioactive agent is built into the array, such that the random deposition of the beads on the surface of the substrate can be "decoded" to allow identification of the bioactive agent at all positions. This may be done in a variety of ways.

20 In a preferred embodiment, the beads are loaded onto the substrate and then the array is decoded, prior to running the assay. This is done by detecting the optical signature associated with the bead at each site on the array. This may be done all at once, if unique optical signatures are used, or sequentially, as is generally outlined above for the "reuse" of sets of optical signatures. Alternatively, decoding may occur after the assay is run..

25 Once made and decoded if necessary, the compositions find use in a number of applications. Generally, a sample containing a target analyte (whether for detection of the target analyte or screening for binding partners of the target analyte) is added to the array, under conditions suitable for binding of the target analyte to at least one of the bioactive agents, i.e. generally physiological conditions. The presence or absence of the target analyte is then detected. As will be appreciated by those in the art, this may be done in a variety of ways, generally through the use of a change in an optical signal. This change can occur via many different mechanisms. A few examples include the binding of a dye-tagged analyte to the bead, the production of a dye species on or near the beads, the

30

destruction of an existing dye species, a change in the optical signature upon analyte interaction with dye on bead, or any other optical interrogatable event.

5 In a preferred embodiment, the change in optical signal occurs as a result of the binding of a target analyte that is labeled, either directly or indirectly, with a detectable label, preferably an optical label such as a fluorochrome. Thus, for example, when a proteinaceous target analyte is used, it may be either directly labeled with a fluor, or indirectly, for example through the use of a labeled antibody. Similarly, nucleic acids are easily labeled with fluorochromes, for example during PCR amplification as is known in the art. Alternatively, upon binding of the target sequences, an intercalating dye (e.g., ethidium bromide) can be added subsequently to signal the presence of the bound target to the probe sequence. Upon binding of the target analyte to a bioactive agent, there is a new optical signal generated at that site, which then may be detected.

Alternatively, in some cases, as discussed above, the target analyte such as an enzyme generates a species (for example, a fluorescent product) that is either directly or indirectly detectable optically.

15 Furthermore, in some embodiments, a change in the optical signature may be the basis of the optical signal. For example, the interaction of some chemical target analytes with some fluorescent dyes on the beads may alter the optical signature, thus generating a different optical signal. For example, fluorophore derivatized receptors may be used in which the binding of the ligand alters the signal.

In a preferred embodiment, sensor redundancy is used. In this embodiment, a plurality of sensor elements, e.g. beads, comprising identical bioactive agents are used. That is, each subpopulation comprises a plurality of beads comprising identical bioactive agents (e.g. binding ligands). By using a number of identical sensor elements for a given array, the optical signal from each sensor element can be combined and any number of statistical analyses run, as outlined below. This can be done for a variety of reasons. For example, in time varying measurements, redundancy can significantly reduce the noise in the system. For non-time based measurements, redundancy can significantly increase the confidence of the data.

20 In a preferred embodiment, a plurality of identical sensor elements are used. As will be appreciated by those in the art, the number of identical sensor elements will vary with the application and use of the sensor array. In general, anywhere from 2 to thousands may be used, with from 2 to 100 being preferred, 2 to 50 being particularly preferred and from 5 to 20 being especially preferred. In general, preliminary results indicate that roughly 10 beads gives a sufficient advantage, although for some applications, more identical sensor elements can be used.

Once obtained, the optical response signals from a plurality of sensor beads within each bead subpopulation can be manipulated and analyzed in a wide variety of ways, including baseline adjustment, averaging, standard deviation analysis, distribution and cluster analysis, confidence interval analysis, mean testing, etc.

- 5 In a preferred embodiment, the first manipulation of the optical response signals is an optional baseline adjustment. In a typical procedure, the standardized optical responses are adjusted to start at a value of 0.0 by subtracting the integer 1.0 from all data points. Doing this allows the baseline-loop data to remain at zero even when summed together and the random response signal noise is canceled out. When the sample is a vapor, the vapor pulse-loop temporal region, however, frequently exhibits a characteristic change in response, either positive, negative or neutral, prior to the vapor pulse and often requires a baseline adjustment to overcome noise associated with drift in the first few data points due to charge buildup in the CCD camera. If no drift is present, typically the baseline from the first data point for each bead sensor is subtracted from all the response data for the same bead. If drift is observed, the average baseline from the first ten data points for each bead sensor is subtracted from all the response data for the same bead. By applying this baseline adjustment, when multiple bead responses are added together they can be amplified while the baseline remains at zero. Since all beads respond at the same time to the sample (e.g. the vapor pulse), they all see the pulse at the exact same time and there is no registering or adjusting needed for overlaying their responses. In addition, other types of baseline adjustment may be done, depending on the requirements and output of the system used.

Once the baseline has been adjusted, although in some embodiments this is not required, a number of possible statistical analyses may be run to generate known statistical parameters. Analyses based on redundancy are known and generally described in texts such as Freund and Walpole, Mathematical Statistics, Prentice Hall, Inc. New Jersey, 1980, hereby incorporated by reference in its entirety.

- 25 In a preferred embodiment, signal summing is done by simply adding the intensity values of all responses at each time point, generating a new temporal response comprised of the sum of all bead responses. These values can be baseline-adjusted or raw. As for all the analyses described herein, signal summing can be performed in real time or during post-data acquisition data reduction and analysis. In one embodiment, signal summing is performed with a commercial spreadsheet program (Excel, Microsoft, Redmond, WA) after optical response data is collected.

In a preferred embodiment, cumulative response data is generated by simply adding all data points in successive time intervals. This final column, comprised of the sum of all data points at a particular

time interval, may then be compared or plotted with the individual bead responses to determine the extent of signal enhancement or improved signal-to-noise ratios as shown in Figs. 14 and 15.

In a preferred embodiment, the mean of the subpopulation (i.e. the plurality of identical beads) is determined, using the well known Equation 1:

5

Equation 1

$$\mu = \sum \frac{x_i}{n}$$

In some embodiments, the subpopulation may be redefined to exclude some beads if necessary (for example for obvious outliers, as discussed below).

In a preferred embodiment, the standard deviation of the subpopulation can be determined, generally using Equation 2 (for the entire subpopulation) and Equation 3 (for less than the entire subpopulation):

10

Equation 2

$$\sigma = \sqrt{\frac{\sum (x_i - \mu)^2}{n}}$$

Equation 3

$$s = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}}$$

As for the mean, the subpopulation may be redefined to exclude some beads if necessary (for example for obvious outliers, as discussed below).

15

In a preferred embodiment, statistical analyses are done to evaluate whether a particular data point has statistical validity within a subpopulation by using techniques including, but not limited to, t distribution and cluster analysis. This may be done to statistically discard outliers that may otherwise skew the result and increase the signal-to-noise ratio of any particular experiment. This may be done using Equation 4:

Equation 4

$$t = \frac{\bar{x} - \mu}{s / \sqrt{n}}$$

In a preferred embodiment, the quality of the data is evaluated using confidence intervals, as is known in the art. Confidence intervals can be used to facilitate more comprehensive data processing to measure the statistical validity of a result.

- 5 In a preferred embodiment, statistical parameters of a subpopulation of beads are used to do hypothesis testing. One application is tests concerning means, also called mean testing. In this application, statistical evaluation is done to determine whether two subpopulations are different. For example, one sample could be compared with another sample for each subpopulation within an array to determine if the variation is statistically significant.
- 10 In addition, mean testing can also be used to differentiate two different assays that share the same code. If the two assays give results that are statistically distinct from each other, then the subpopulations that share a common code can be distinguished from each other on the basis of the assay and the mean test, shown below in Equation 5:

Equation 5

$$z = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}}}$$

- 15 Furthermore, analyzing the distribution of individual members of a subpopulation of sensor elements may be done. For example, a subpopulation distribution can be evaluated to determine whether the distribution is binomial, Poisson, hypergeometric, etc.

- 20 In addition to the sensor redundancy, a preferred embodiment utilizes a plurality of sensor elements that are directed to a single target analyte but yet are not identical. For example, a single target nucleic acid analyte may have two or more sensor elements each comprising a different probe. This adds a level of confidence as non-specific binding interactions can be statistically minimized. When nucleic acid target analytes are to be evaluated, the redundant nucleic acid probes may be overlapping, adjacent, or spatially separated. However, it is preferred that two probes do not compete for a single binding site, so adjacent or separated probes are preferred. Similarly, when proteinaceous

target analytes are to be evaluated, preferred embodiments utilize bioactive agent binding agents that bind to different parts of the target. For example, when antibodies (or antibody fragments) are used as bioactive agents for the binding of target proteins, preferred embodiments utilize antibodies to different epitopes.

- 5 In this embodiment, a plurality of different sensor elements may be used, with from about 2 to about 20 being preferred, and from about 2 to about 10 being especially preferred, and from 2 to about 5 being particularly preferred, including 2, 3, 4 or 5. However, as above, more may also be used, depending on the application.

As above, any number of statistical analyses may be run on the data from target redundant sensors.

- 10 One benefit of the sensor element summing (referred to herein as "bead summing" when beads are used), is the increase in sensitivity that can occur. Detection limits in the zeptomole range can be observed.

- As will be appreciated by those in the art, in some embodiments, the presence or absence of the target analyte may be done using changes in other optical or non-optical signals, including, but not  
15 limited to, surface enhanced Raman spectroscopy, surface plasmon resonance, radioactivity, etc.

- The assays may be run under a variety of experimental conditions, as will be appreciated by those in the art. A variety of other reagents may be included in the screening assays. These include reagents like salts, neutral proteins, e.g. albumin, detergents, etc which may be used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Also reagents that  
20 otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used. The mixture of components may be added in any order that provides for the requisite binding. Various blocking and washing steps may be utilized as is known in the art.

- In a preferred embodiment, the compositions are used to probe a sample solution for the presence or  
25 absence of a target analyte. By "target analyte" or "analyte" or grammatical equivalents herein is meant any atom, molecule, ion, molecular ion, compound or particle to be either detected or evaluated for binding partners. As will be appreciated by those in the art, a large number of analytes may be used in the present invention; basically, any target analyte can be used which binds a bioactive agent or for which a binding partner (i.e. drug candidate) is sought.



Suitable analytes include organic and inorganic molecules, including biomolecules. When detection of a target analyte is done, suitable target analytes include, but are not limited to, an environmental pollutant (including pesticides, insecticides, toxins, etc.); a chemical (including solvents, polymers, organic materials, etc.); therapeutic molecules (including therapeutic and abused drugs, antibiotics, etc.); biomolecules (including hormones, cytokines, proteins, nucleic acids, lipids, carbohydrates, cellular membrane antigens and receptors (neural, hormonal, nutrient, and cell surface receptors) or their ligands, etc); whole cells (including procaryotic (such as pathogenic bacteria) and eukaryotic cells, including mammalian tumor cells); viruses (including retroviruses, herpesviruses, adenoviruses, lentiviruses, etc.); and spores; etc. Particularly preferred analytes are nucleic acids and proteins.

10 In a preferred embodiment, the target analyte is a protein. As will be appreciated by those in the art, there are a large number of possible proteinaceous target analytes that may be detected or evaluated for binding partners using the present invention. Suitable protein target analytes include, but are not limited to, (1) immunoglobulins; (2) enzymes (and other proteins); (3) hormones and cytokines (many of which serve as ligands for cellular receptors); and (4) other proteins.

15 In a preferred embodiment, the target analyte is a nucleic acid. These assays find use in a wide variety of applications.

In a preferred embodiment, the probes are used in genetic diagnosis. For example, probes can be made using the techniques disclosed herein to detect target sequences such as the gene for nonpolyposis colon cancer, the BRCA1 breast cancer gene, P53, which is a gene associated with a variety of cancers, the Apo E4 gene that indicates a greater risk of Alzheimer's disease, allowing for easy presymptomatic screening of patients, mutations in the cystic fibrosis gene, or any of the others well known in the art.

20 In an additional embodiment, viral and bacterial detection is done using the complexes of the invention. In this embodiment, probes are designed to detect target sequences from a variety of bacteria and viruses. For example, current blood-screening techniques rely on the detection of anti-HIV antibodies. The methods disclosed herein allow for direct screening of clinical samples to detect HIV nucleic acid sequences, particularly highly conserved HIV sequences. In addition, this allows direct monitoring of circulating virus within a patient as an improved method of assessing the efficacy of anti-viral therapies. Similarly, viruses associated with leukemia, HTLV-I and HTLV-II, may be detected in this way. Bacterial infections such as tuberculosis, chlamydia and other sexually transmitted diseases, may also be detected.

In a preferred embodiment, the nucleic acids of the invention find use as probes for toxic bacteria in the screening of water and food samples. For example, samples may be treated to lyse the bacteria to release its nucleic acid, and then probes designed to recognize bacterial strains, including, but not limited to, such pathogenic strains as, *Salmonella*, *Campylobacter*, *Vibrio cholerae*, *Leishmania*, enterotoxigenic strains of *E. coli*, and Legionnaire's disease bacteria. Similarly, bioremediation strategies may be evaluated using the compositions of the invention.

In a further embodiment, the probes are used for forensic "DNA fingerprinting" to match crime-scene DNA against samples taken from victims and suspects.

In an additional embodiment, the probes in an array are used for sequencing by hybridization.

The present invention also finds use as a methodology for the detection of mutations or mismatches in target nucleic acid sequences. For example, recent focus has been on the analysis of the relationship between genetic variation and phenotype by making use of polymorphic DNA markers. Previous work utilized short tandem repeats (STRs) as polymorphic positional markers; however, recent focus is on the use of single nucleotide polymorphisms (SNPs), which occur at an average frequency of more than 1 per kilobase in human genomic DNA. Some SNPs, particularly those in and around coding sequences, are likely to be the direct cause of therapeutically relevant phenotypic variants. There are a number of well known polymorphisms that cause clinically important phenotypes; for example, the apoE2/3/4 variants are associated with different relative risk of Alzheimer's and other diseases (see Cordor et al., Science 261(1993). Multiplex PCR amplification of SNP loci with subsequent hybridization to oligonucleotide arrays has been shown to be an accurate and reliable method of simultaneously genotyping at least hundreds of SNPs; see Wang et al., Science, 280:1077 (1998); see also Schafer et al., Nature Biotechnology 16:33-39 (1998). The compositions of the present invention may easily be substituted for the arrays of the prior art.

In a preferred embodiment, the compositions of the invention are used to screen bioactive agents to find an agent that will bind, and preferably modify the function of, a target molecule. As above, a wide variety of different assay formats may be run, as will be appreciated by those in the art. Generally, the target analyte for which a binding partner is desired is labeled; binding of the target analyte by the bioactive agent results in the recruitment of the label to the bead, with subsequent detection.

In a preferred embodiment, the binding of the bioactive agent and the target analyte is specific; that is, the bioactive agent specifically binds to the target analyte. By "specifically bind" herein is meant that the agent binds the analyte, with specificity sufficient to differentiate between the analyte and other components or contaminants of the test sample. However, as will be appreciated by those in the art, it

will be possible to detect analytes using binding which is not highly specific; for example, the systems may use different binding ligands, for example an array of different ligands, and detection of any particular analyte is via its "signature" of binding to a panel of binding ligands, similar to the manner in which "electronic noses" work. This finds particular utility in the detection of chemical analytes. The binding should be sufficient to remain bound under the conditions of the assay, including wash steps to remove non-specific binding, although in some embodiments, wash steps are not desired; i.e. for detecting low affinity binding partners. In some embodiments, for example in the detection of certain biomolecules, the dissociation constants of the analyte to the binding ligand will be less than about  $10^{-4}$ - $10^{-6}$  M<sup>-1</sup>, with less than about  $10^{-5}$  to  $10^{-9}$  M<sup>-1</sup> being preferred and less than about  $10^{-7}$ - $10^{-9}$  M<sup>-1</sup> being particularly preferred.

The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All references cited herein are incorporated by reference in their entirety.

## EXAMPLES

### Example 1: Enzyme-Based Sensor

#### Subpopulation A

Bioactive agent: Alkaline phosphatase

Target substrate: fluorescein diphosphate (FDP)

Reported dye ratio: 1:1 ratio of DiIC:TRC, where DiIC is 1,1',3,3',3'-hexamethyl-indodicarbocyanine iodide and TRC is Texas Red cadaverine

A range of ratios of light intensities are selected that are representative of the optical signature for the dye ratio of the subpopulation based on the quantum yield of the two dyes. The optical signature for this subpopulation is:

$$\frac{\text{DiIC } \lambda \text{ intensity-ave. DiIC background}}{\text{TRC } \lambda \text{ intensity-ave. TRC background}} = 0.847 \pm 0.23$$

#### Subpopulation B

Bioactive agent: B-Galactosidase;

Target substrate = fluorescein di-B-galactopyranoside (FDG)

Reporter dye ratio: 10:1 ratio of DiIC:TRC which translates to an optical signature of:

$$\frac{\text{DiIC } \lambda \text{ intensity-ave. DiIC background}}{\text{TRC } \lambda \text{ intensity-ave. TRC background}} = 4.456 \pm 1.27$$

Subpopulation C

Bioactive agent: B-glucuronidase

Target substrate = fluorescein di-B-D-glucuronide (FDGicu).

Reporter dye ratio: 1:10 ratio of DiIC:TRC, which translates to an optical signature of:

$$\frac{\text{DiIC A intensity-ave. DiIC background}}{\text{TRC A intensity-ave. TRC background}} = 0.2136 + 0.03$$

When the microsphere populations are in the presence of one or more of the substrates, the respective enzymes on the microspheres catalyze the breakdown of the substrates producing fluorescein which is fluorescent, emitting light at 530 nanometers when excited at 490 nm. The production of fluorescein localized to particular beads is then monitored. In this approach, the localization of fluorescein around the microspheres is increased by using a substrate solution of 90% glycerol and 10% substrate. The glycerol inhibits the generated fluorescein from diffusing away from the microsphere reaction sites.

During the experiment, images in the encoded wavelengths are first taken. Since both DiIC and TRC are excited at 577 nm. Each microsphere's emissions at 670 nm, indicative of the presence of DiIC and 610 nm indicative of the presence of TRC were recorded using a 595 nm dichroic and an acquisition time of 5 seconds for the CCD 236. Next, the distal end 212 of the fiber bundle is placed in a buffer and another image taken while illuminating the beams with 490 nm light. Emissions in the 530 nm fluorescein wavelengths were recorded with a 505 nm dichroic. In this case, a CCD acquisition time of one second was used. This process provides a background normalizing image. The buffer was removed and the fiber allowed to dry to avoid substrate solution dilution.

The substrate solution is then introduced and CCD images acquired every 30 seconds to a minute for 30 minutes While illuminating the microspheres with 490 nm light and collecting emissions in the 530 nm range. Fiber is then placed back in the buffer solution and another background image captured.

Those beads that generate a signal indicative of fluorescein production are decoded. Depending in the ratio of the intensity of light from the two reporter dyes, DiIC:TRC, the bioactive agent of the optically active beads may be decoded according to the following table.

0.617 - 1.08	alkaline phosphatase bead
3.188-5.725	$\beta$ -galactosidase bead
0.183 - 0.243	$\beta$ -glucunonidese bead

This process is then repeated for the remaining two substrates.

Figs. 8A-8C are images generated by the CCD 236 when the bead populations are exposed to fluorescein diphosphate. Fig. 8A illustrates the signals from the alkaline phosphatase microspheres when excited at 490 nm and recording emissions at 530 nm, emissions at this wavelength being indicative of fluorescein production. Fig. 8B shows the image captured by the CCD when the microspheres are excited at 577 nm and emissions at 670 nm are recorded. This wavelength is an encoding wavelength indicative of the concentration of DiIC on the microspheres. Finally, Fig. 8C shows the image when the microspheres are excited with 577 nm light and emissions in the 610 nm range are recorded being indicative of the concentration of TRC in the microspheres.

In a similar vein, Figs. 9A and 9B are images when the microspheres are exposed to fluorescein  $\beta$ -D-galactosidase. Fig. 9A shows emissions at 530 nm indicative of the fluorescein production; and Fig. 9B shows light emitted at the 670 nm range indicative of the presence of DiIC.

These micrographs, Fig. 8A-8C and 9A-9B illustrate that fluorescein production around the microspheres may be detected as an optical signature change indicative of reactions involving the bioactive agent of the microspheres. The micrographs also illustrate that the optical signatures may be decoded to determine the chemical functionalities on each microsphere.

#### Immunosensor

Three separate subpopulations of beads were used. In subpopulation A, rabbit antibodies (Ab) were affixed to the surface of the microspheres; in subpopulation B, goat antibodies were affixed to the microspheres; and in subpopulation C, mouse antibodies were affixed to the microspheres. These three separate subpopulations were identified using a DiIC:TRC encoding ratio similar to that in the previously described experiment.

For the first step of the experiment, images at the encoded wavelengths were captured using 577 nm excitation and looking for emissions at 610 and 670 nm. After this decoding, the fiber was placed in a buffer and an image taken at 530 nm with 490 nm excitation. This provided a background normalizing signal at the fluorescein emission wavelength. Next, the fiber was placed in rabbit IgG antigen (Ag) which is fluorescein labeled. Images were then captured every few minutes at the 530 nm emission wavelength for fluorescein. Figs. IOA and IOB are micrographs showing the image captured by the CCD prior to and subsequent to exposure to a rabbit antigen, which clearly show reaction of the selected microspheres within the population.

Note, if the fluorescein background from the antigen solution is too high to see the antibody-antigen signal, the fiber bundle may be placed in a buffer. This removes the background fluorescence leaving only the Ab-Ag signal.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

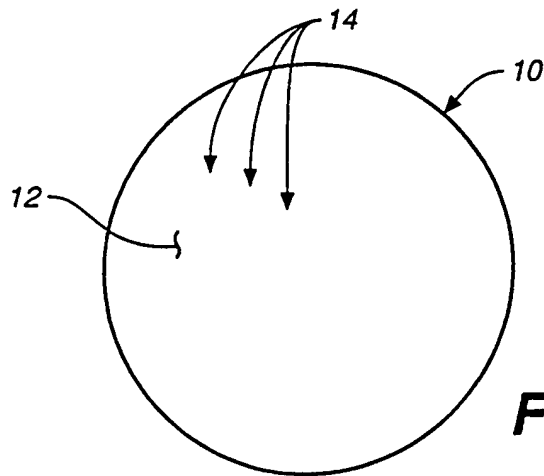
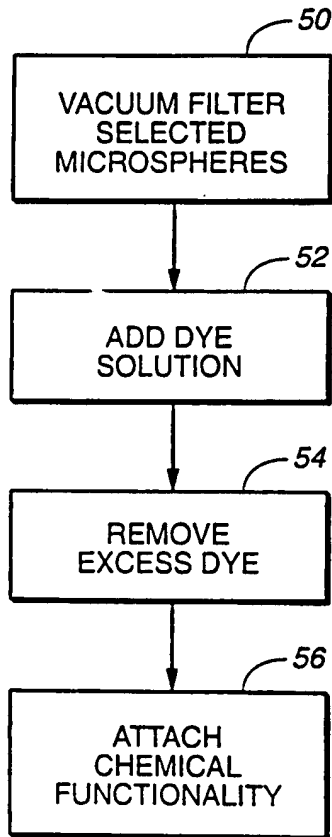
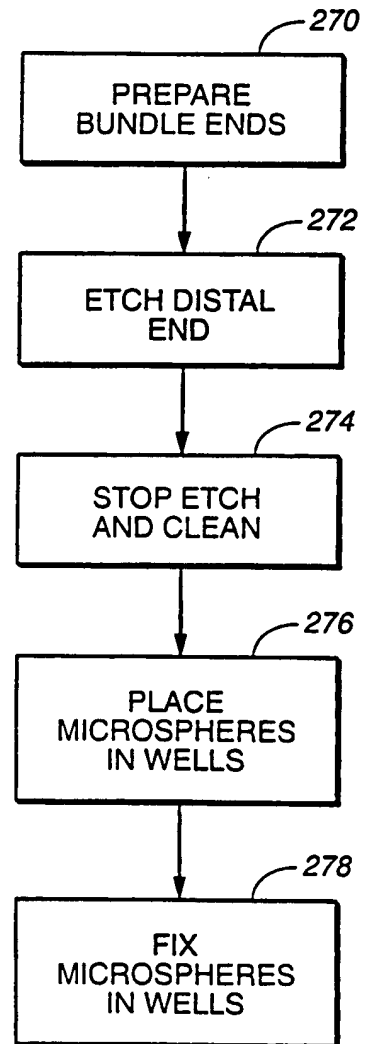
## CLAIMS

We claim:

1. A composition comprising:
  - a) a substrate with a surface comprising discrete sites, wherein said substrate is not a fiber optic bundle; and
  - b) a population of microspheres comprising at least a first and a second subpopulation each comprising:
    - i) a first and a second bioactive agent, respectively; and
    - ii) a first and a second optical signature, respectively, capable of identifying said bioactive agent;wherein said microspheres are distributed on said sites.
2. A composition according to claim 1 wherein said sites comprise wells.
3. A composition according to claim 1 or 2 wherein said substrate is glass.
4. A composition according to claim 1, 2 or 3 wherein said substrate is plastic.
5. A composition according to claim 1, 2, 3 or 4 wherein said sites comprise chemically functionalized sites.
6. A composition according to claim 1, 2, 3, 4 or 5 wherein said bioactive agents comprise nucleic acids.
7. A composition according to claim 1, 2, 3, 4 or 5 wherein said bioactive agents comprise proteins.
8. A composition according to claim 7 wherein said proteins are selected from the group consisting of enzymes and antibodies.
9. A composition according to claim 1, 2, 3, 4, 5, 6, 7 or 8 wherein said at least one of said optical signatures comprises at least one fluorescent dye.
10. A composition according to claim 1, 2, 3, 4, 5, 6, 7, 8 or 9 wherein at least one of said optical signatures comprises at least two fluorescent dyes.

11. A method of determining the presence of a target analyte in a sample comprising:
- a) contacting said sample with a composition comprising:
    - i) a substrate with a surface comprising discrete sites, wherein said substrate is not a fiber optic bundle; and
    - ii) a population of microspheres comprising at least a first and a second subpopulation each comprising:
      - 1) a bioactive agent; and
      - 2) an optical signature capable of identifying said bioactive agent;wherein said microspheres are distributed on said surface; and
  - b) determining the presence or absence of said target analyte.
12. A method according to claim 11 further comprising identifying the location of each bioactive agent on said substrate.
13. A method of making a composition comprising:
- a) forming a surface comprising individual sites on a substrate, wherein said substrate is not a fiber optic bundle; and
  - b) distributing microspheres on said surface such that said individual sites contain microspheres, wherein said microspheres comprise at least a first and a second subpopulations each comprising:
    - i) a bioactive agent; and
    - ii) an optical signature capable of identifying said bioactive agent.
14. A method according to claim 13 wherein said distributing comprises serially adding said subpopulations to said sites.
15. A method according to claim 11, 12 or 13 wherein said bioactive agents are nucleic acids.
16. A method according to claim 12 or 13 wherein said bioactive agents are proteins.
17. A method according to claim 11, 12, 13, 14, 15 or 16 wherein said sites are wells.



**FIG.\_1****FIG.\_2****FIG.\_6**

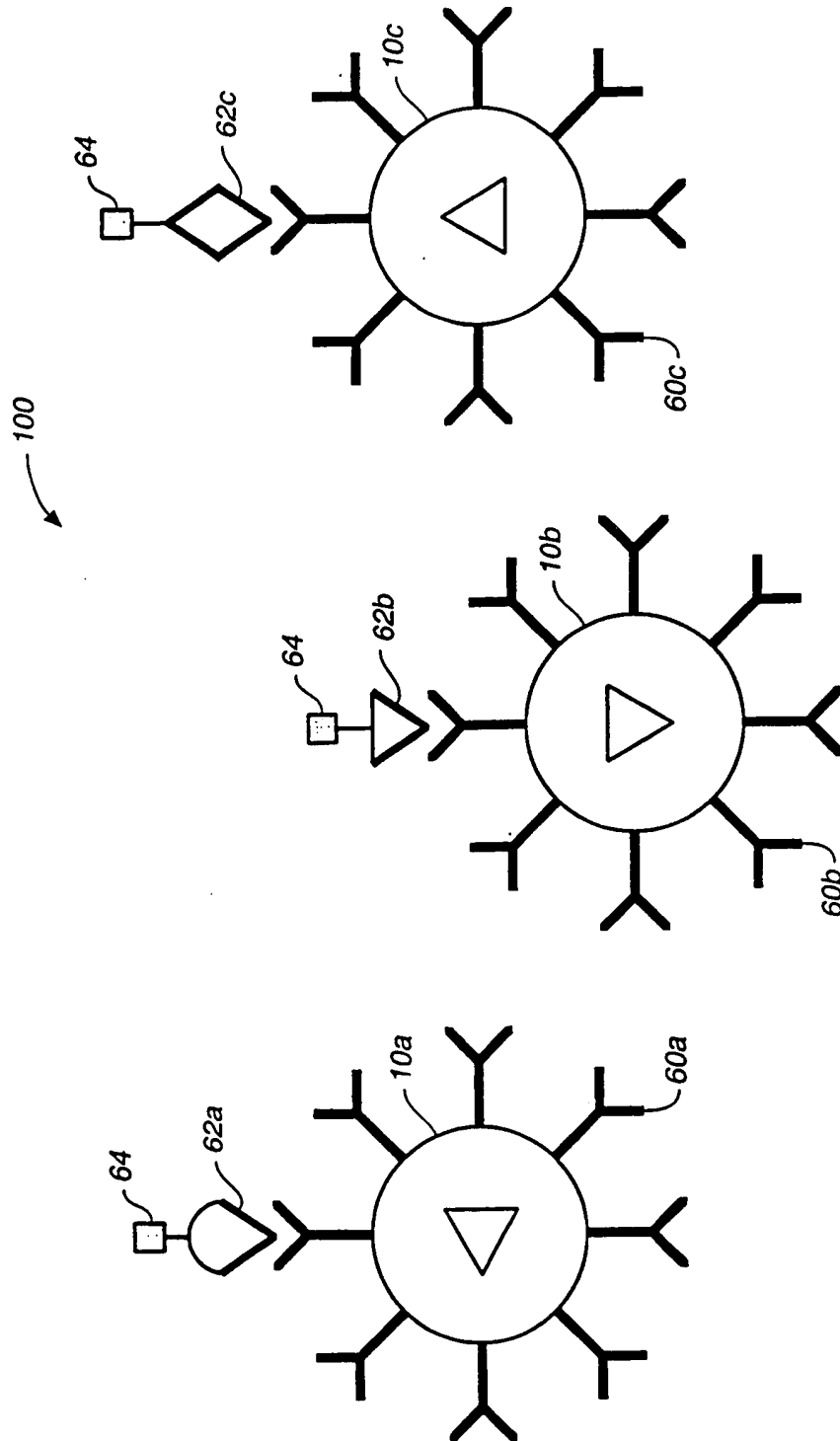
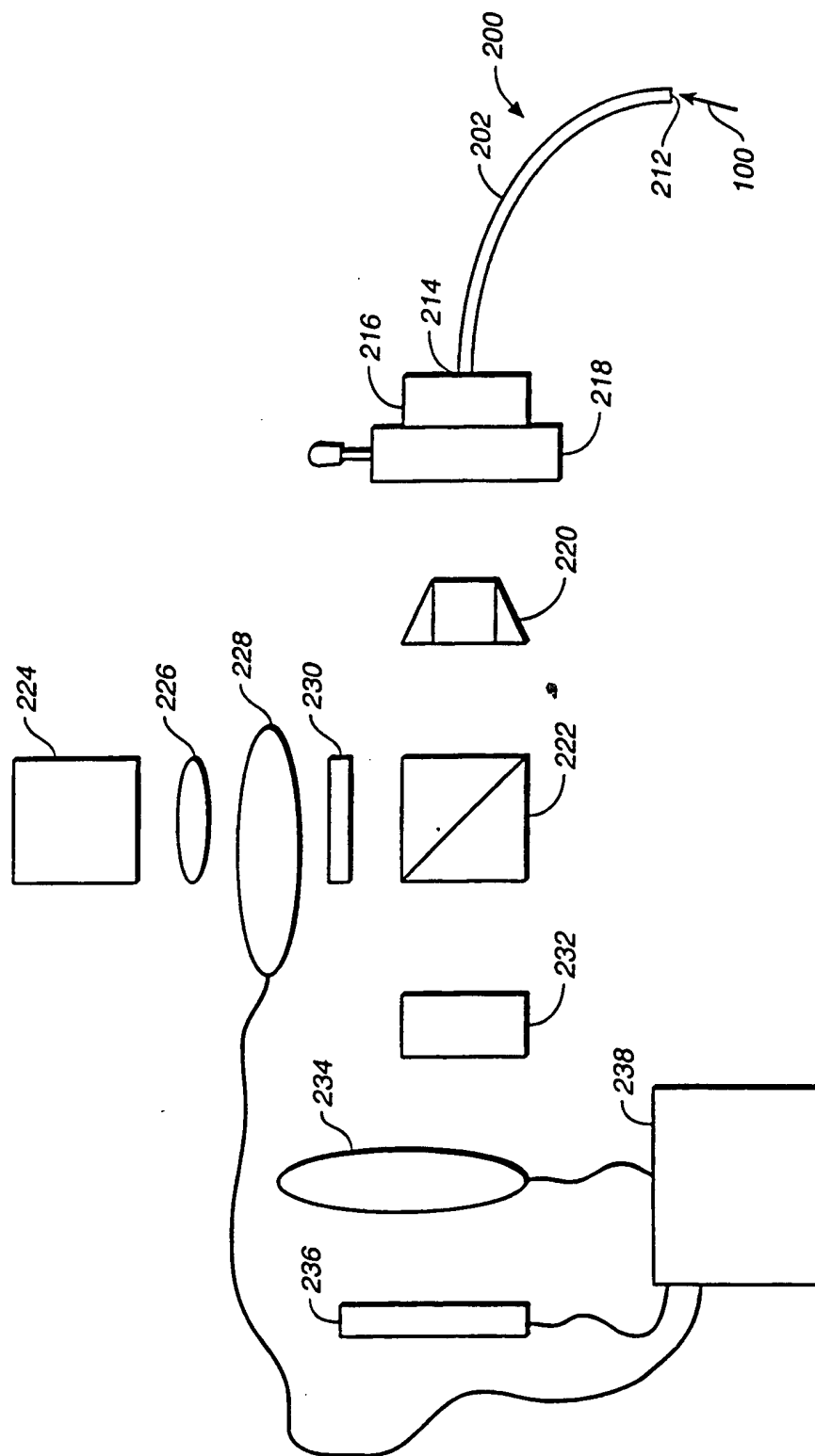


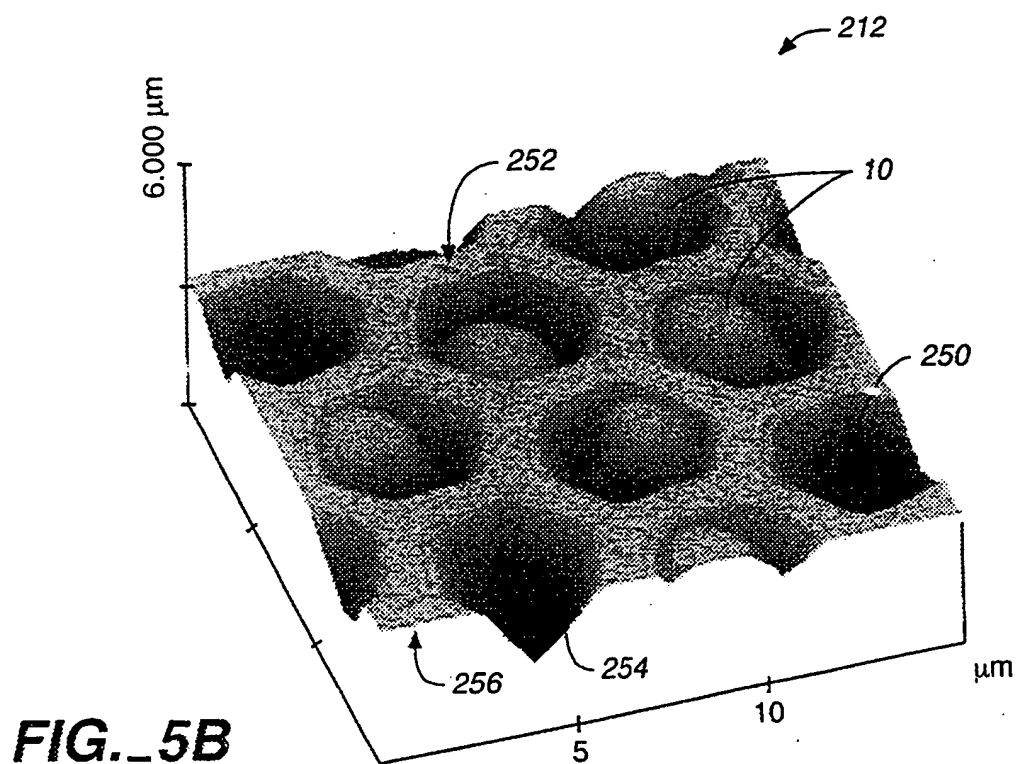
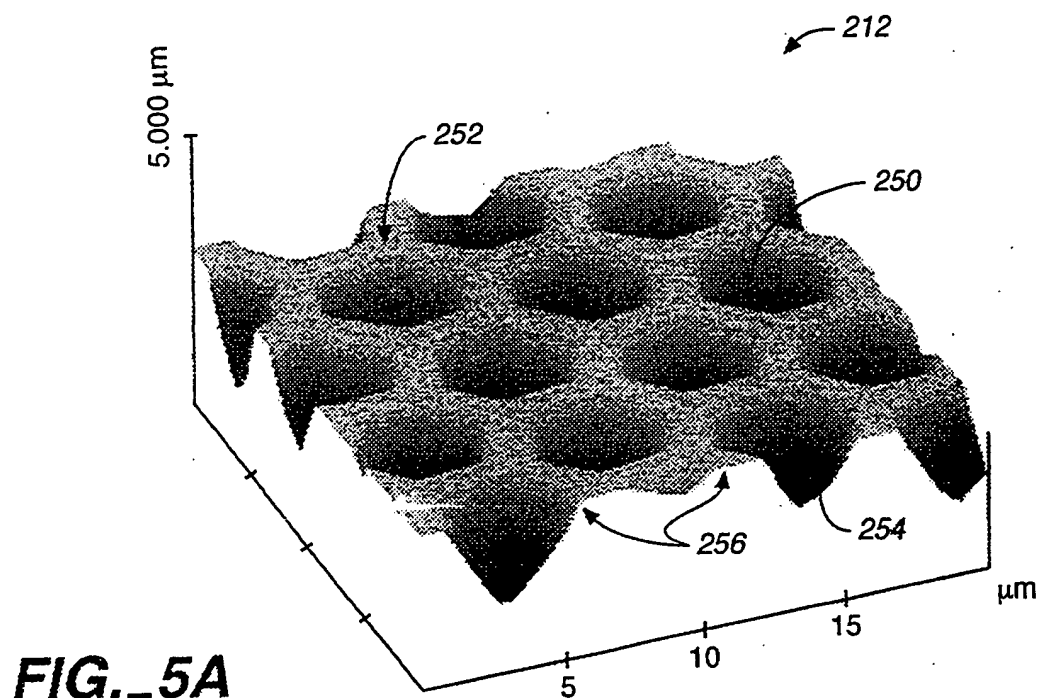
FIG. 3

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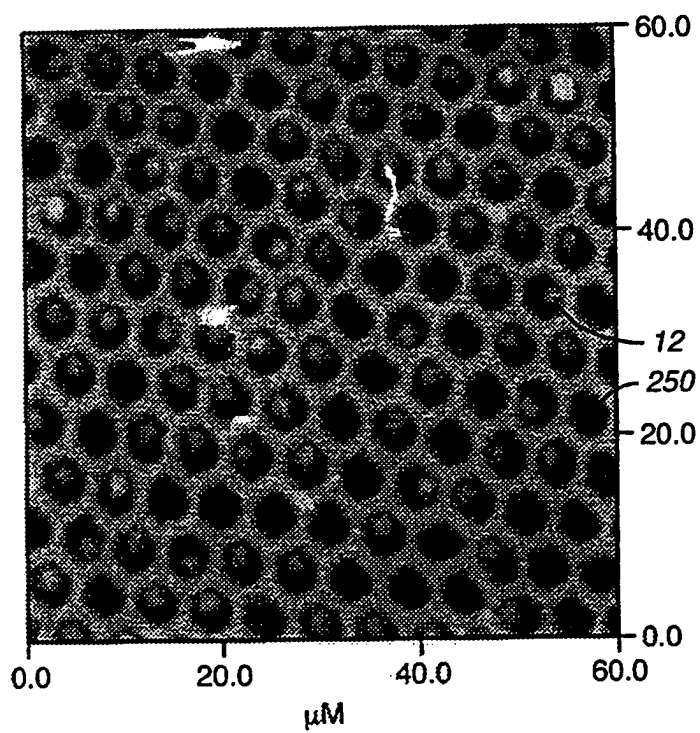
**FIG. 4**

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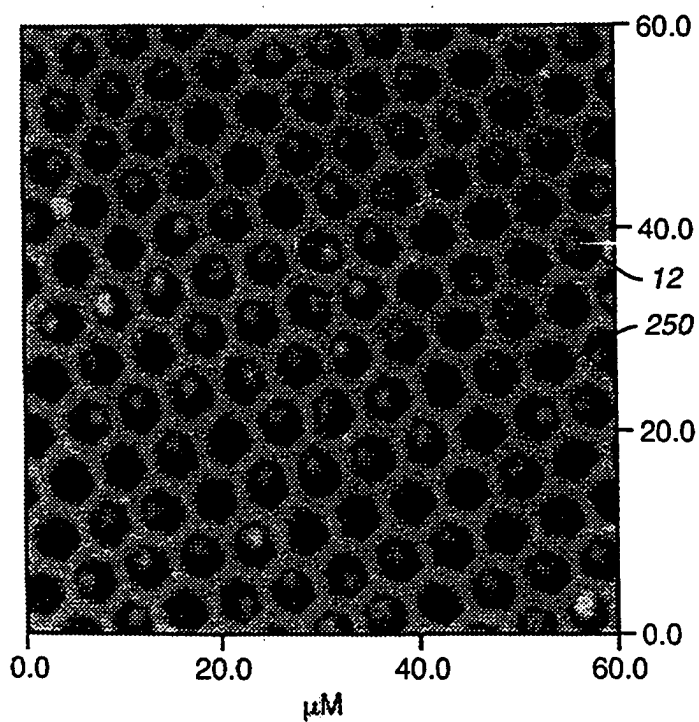


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**FIG.\_7A**

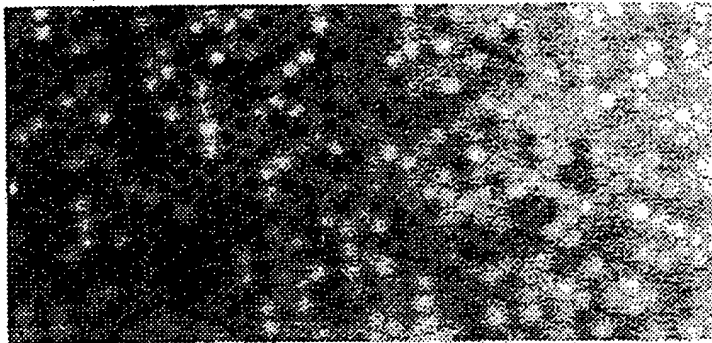


**FIG.\_7B**

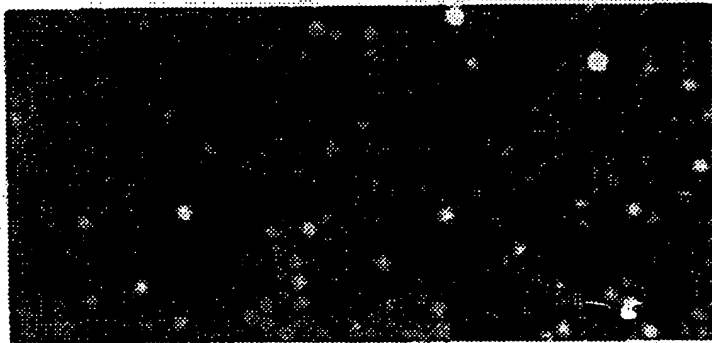


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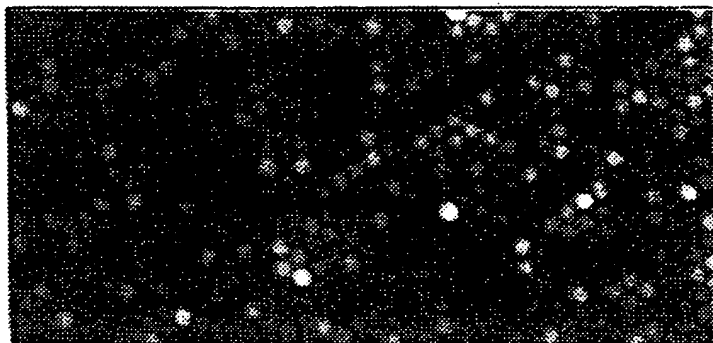
**FIG.\_8A**



**FIG.\_8B**

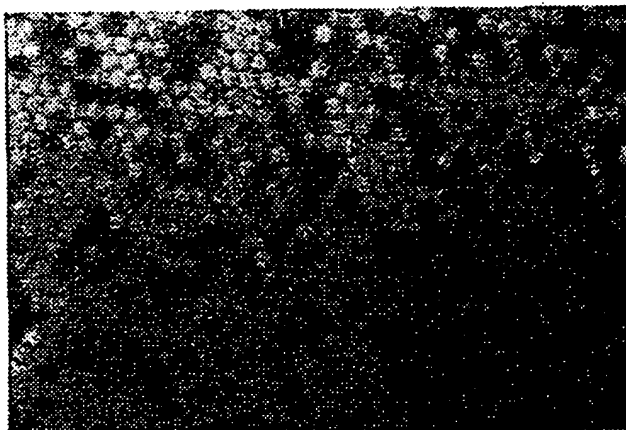


**FIG.\_8C**

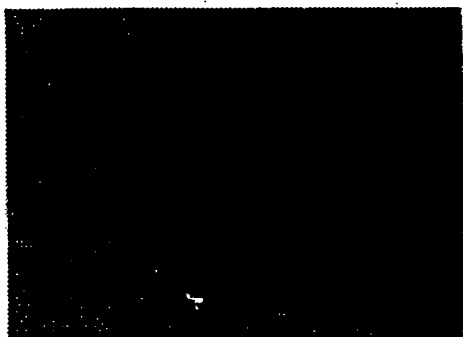
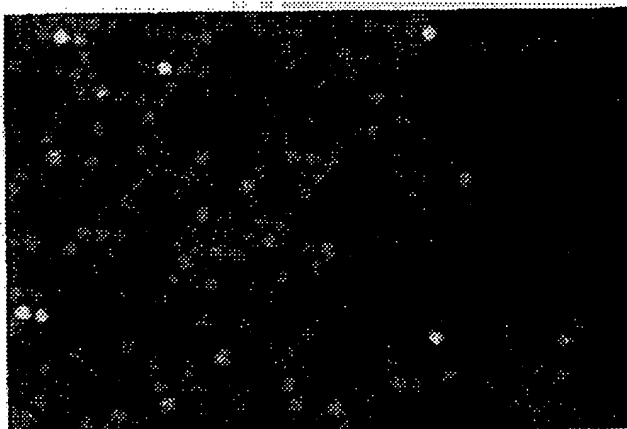


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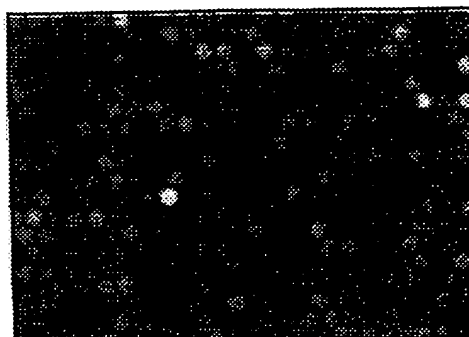
**FIG.\_9A**



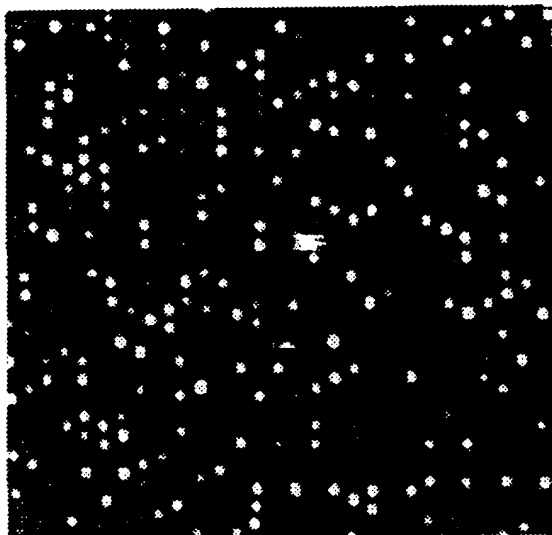
**FIG.\_9B**



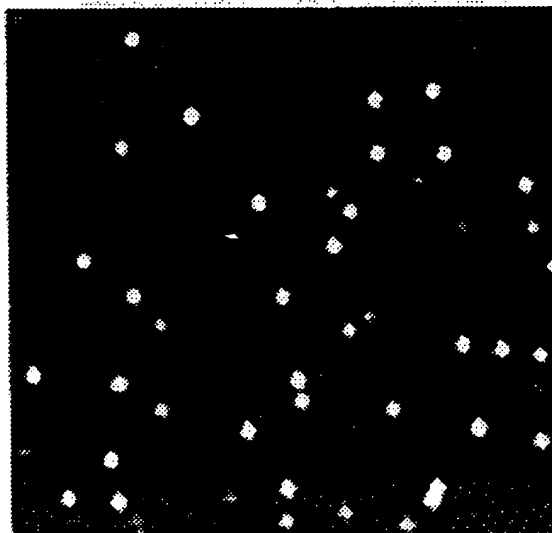
**FIG.\_10A**



**FIG.\_10B**



**FIG. 11A**

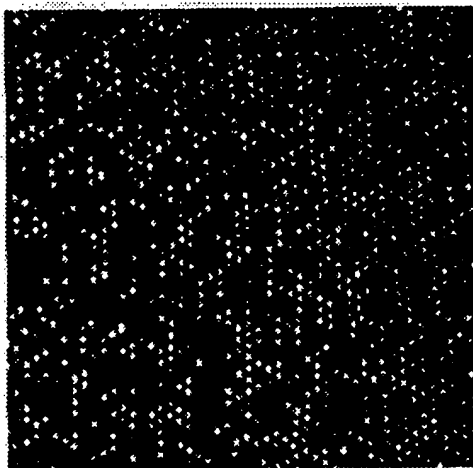


**FIG. 11B**

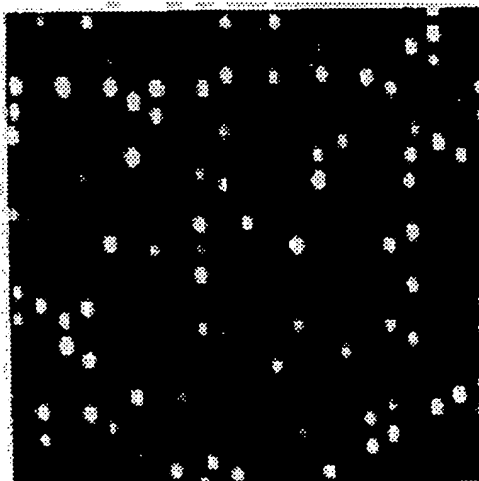


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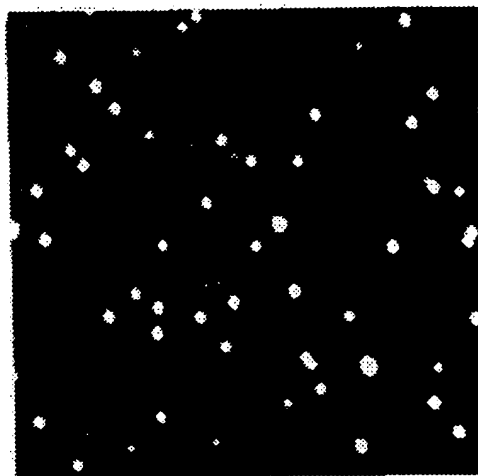
Mean: 143.8  
S.D.: 39.4  
C.V.: 27.3%

**FIG.\_12A**

Mean: 167.3  
S.D.: 45.7  
C.V.: 27.3%

**FIG.\_12B**

Mean: 198.9  
S.D.: 38.3  
C.V.: 19.3%

**FIG.\_12C**

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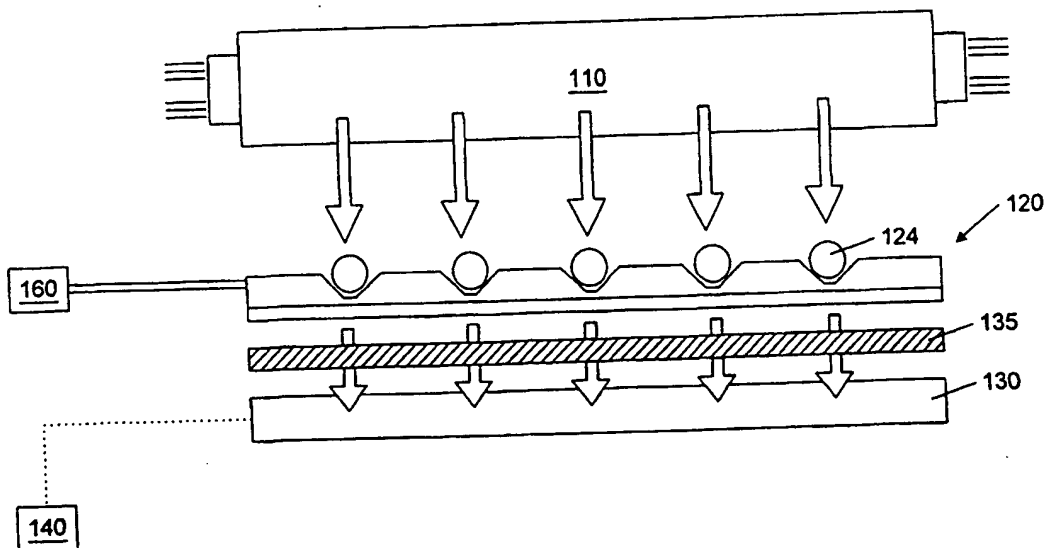
(51) International Patent Classification <sup>7</sup> : <b>G01N 21/64, 21/77</b>		A1	(11) International Publication Number: <b>WO 00/04372</b>
			(43) International Publication Date: 27 January 2000 (27.01.00)
(21) International Application Number: PCT/US99/16162		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 16 July 1999 (16.07.99)			
(30) Priority Data: 60/093,111 16 July 1998 (16.07.98) US 09/287,248 7 April 1999 (07.04.99) US			
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**Published**

*With international search report.*

*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: SENSOR ARRAYS FOR THE MEASUREMENT AND IDENTIFICATION OF MULTIPLE ANALYTES IN SOLUTIONS



(57) Abstract

A system for the rapid characterization of multi-analyte fluids, in one embodiment, includes a light source, a sensor array, and a detector. The sensor array is formed from a supporting member into which a plurality of cavities may be formed. A series of chemically sensitive particles microspheres are, in one embodiment positioned within the cavities. The particles may be configured to produce a signal when a receptor coupled to the particle interacts with the analyte. Using pattern recognition techniques, the analytes within a multi-analyte fluid may be characterized.

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**TITLE: SENSOR ARRAYS FOR THE MEASUREMENT AND IDENTIFICATION OF MULTIPLE ANALYTES IN SOLUTIONS**

**STATEMENT REGARDING FEDERALLY SPONSORED  
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Research leading to this invention was federally supported, in part, by grant No. 1R01GM57306-01 entitled "The Development of an Electronic Tongue" from the National Institute of Health and the U.S.

Government has certain rights to this invention.

**BACKGROUND OF THE INVENTION**

**1. Field of the Invention**

The present invention relates to a method and device for the detection of analytes in a fluid. More particularly, the invention relates to the development of a sensor array system capable of discriminating mixtures of analytes, toxins, and/or bacteria in medical, food/beverage, and environmental solutions.

**2. Brief Description of the Related Art**

The development of smart sensors capable of discriminating different analytes, toxins, and bacteria has become increasingly important for clinical, environmental, health and safety, remote sensing, military, food/beverage and chemical processing applications. Although many sensors capable of high sensitivity and high selectivity detection have been fashioned for single analyte detection, only in a few selected cases have array sensors been prepared which display solution phase multi-analyte detection capabilities. The advantages of such array systems are their utility for the analysis of multiple analytes and their ability to be "trained" to respond to new stimuli. Such on site adaptive analysis capabilities afforded by the array structures make their utilization promising for a variety of future applications. Array based sensors displaying the capacity to sense and identify complex vapors have been demonstrated recently using a number of distinct transduction schemes. For example, functional sensors based on Surface Acoustic Wave (SAW), tin oxide (SnO<sub>2</sub>) sensors, conductive organic polymers, and carbon black/polymer composites have been fashioned. The use of tin oxide sensors, for example, is described in U.S. Patent No. 5,654,497 to Hoffheins et al. These sensors display the capacity to identify and discriminate between a variety of organic vapors by virtue of small site-to-site differences in response characteristics. Pattern recognition of the overall fingerprint response for the array serves as the basis for an olfaction-like detection of the vapor phase analyte species. Indeed, several commercial "electronic noses" have been developed recently. Most of the well established sensing elements are based on SnO<sub>2</sub> arrays which have been derivatized so as to yield chemically distinct response properties. Arrays based on SAW crystals yield extremely sensitive responses to vapor, however, engineering challenges have prevented the creation of large SAW arrays having multiple sensor sites. To our knowledge, the largest SAW device reported to date possesses only 12 sensor elements. Additionally, limited chemical diversity and the lack of understanding of the molecular features of such systems makes their expansion into more complex analysis difficult.

Other structures have been developed that are capable of identifying and discriminating volatile organic molecules. One structure involves a series of conductive polymer layers deposited onto metal contacting layers. When these sensors are exposed to volatile reagents, some of the volatile reagents adsorb into the polymer layers, leading to small changes in the electrical resistance of these layers. It is the small differences in the behavior of the various sites that allows for a discrimination, identification, and quantification of the vapors. The detection process takes only a few seconds, and sensitivities of part-per-billion can be achieved with this relatively simple approach. This "electronic nose" system is described in U.S. Patent No. 5,698,089 to Lewis et al. which is incorporated by reference as if set forth herein.

Although the above described electronic nose provides an impressive capability for monitoring volatile reagents, the system possesses a number of undesirable characteristics that warrant the development of alternative sensor array systems. For example, the electronic nose can be used only for the identification of volatile reagents. For many environmental, military, medical, and commercial applications, the identification and quantification of analytes present in liquid or solid-phase samples is necessary. Moreover, the electronic nose systems are expensive (e.g., the Aromascan system costs about \$50,000/unit) and bulky ( $\geq 1 \text{ ft}^3$ ). Furthermore, the functional elements for the currently available electronic nose are composed of conductive polymer systems which possess little chemical selectivity for many of the analytes which are of interest to the military and civilian communities.

One of the most commonly employed sensing techniques has exploited colloidal polymer microspheres for latex agglutination tests (LATs) in clinical analysis. Commercially available LATs for more than 60 analytes are used routinely for the detection of infectious diseases, illegal drugs, and early pregnancy tests. The vast majority of these types of sensors operate on the principle of agglutination of latex particles (polymer microspheres) which occurs when the antibody-derivatized microspheres become effectively "cross-linked" by a foreign antigen resulting in the attachment to, or the inability to pass through a filter. The dye-doped microspheres are then detected colorimetrically upon removal of the antigen carrying solution. However, the LATs lack the ability to be utilized for multiple, real time analyte detection schemes as the nature of the response intrinsically depends on a cooperative effect of the entire collection of microspheres.

Similar to the electronic nose, array sensors that have shown great analytical promise are those based on the "DNA on a chip" technology. These devices possess a high density of DNA hybridization sites that are affixed in a two-dimensional pattern on a planar substrate. To generate nucleotide sequence information, a pattern is created from unknown DNA fragments binding to various hybridization sites. Both radiochemical and optical methods have provided excellent detection limits for analysis of limited quantities of DNA. (Stimpson, D. I.; Hoijer, J. V.; Hsieh, W.; Jou, C.; Gardon, J.; Theriault, T.; Gamble, R.; Baldeschwieler, J.D. Proc. Natl. Acad. Sci. USA 1995, 92, 6379). Although quite promising for the detection of DNA fragments, these arrays are generally not designed for non-DNA molecules, and accordingly show very little sensitivity to smaller organic molecules. Many of the target molecules of interest to civilian and military communities, however, do not possess DNA components. Thus, the need for a flexible, non-DNA based sensor is still desired. Moreover, while a number of prototype DNA chips containing up to a few thousand different nucleic acid probes have been described, the existing technologies tend to be difficult to expand to a practical size. As a result, DNA chips may be prohibitively expensive for practical uses.

A system of analyzing fluid samples using an array formed of heterogeneous, semi-selective thin films

which function as sensing receptor units is described in U.S. Patent No. 5,512,490 to Walt et al., which is incorporated by reference as if set forth herein. Walt appears to describe the use of covalently attached polymeric "cones" which are grown via photopolymerization onto the distal face of fiber optic bundles. These sensor probes appear to be designed with the goal of obtaining unique, continuous, and reproducible responses from small localized regions of dye-doped polymer. The polymer appears to serve as a solid support for indicator molecules that provide information about test solutions through changes in optical properties. These polymer supported sensors have been used for the detection of analytes such as pH, metals, and specific biological entities. Methods for manufacturing large numbers of reproducible sensors, however, has yet to be developed. Moreover, no methods for acquisitions of data streams in a simultaneous manner are commercially available with this system.

Optical alignment issues may also be problematic for these systems.

A method of rapid sample analysis for use in the diagnostic microbiology field is also desirable. The techniques now used for rapid microbiology diagnostics detect either antigens or nucleic acids. Rapid antigen testing is based on the use of antibodies to recognize either the single cell organism or the presence of infected cell material. Inherent to this approach is the need to obtain and characterize the binding of the antibody to unique structures on the organism being tested. Since the identification and isolation of the appropriate antibodies is time consuming, these techniques are limited to a single agent per testing module and there is no opportunity to evaluate the amount of agent present.

Most antibody methods are relatively insensitive and require the presence of  $10^5$  to  $10^7$  organisms. The response time of antibody-antigen reactions in diagnostic tests of this type ranges from 10 to 120 minutes, depending on the method of detection. The fastest methods are generally agglutination reactions, but these methods are less sensitive due to difficulties in visual interpretation of the reactions. Approaches with slower reaction times include antigen recognition by antibody conjugated to either an enzyme or chromophore. These test types tend to be more sensitive, especially when spectrophotometric methods are used to determine if an antigen-antibody reaction has occurred. These detection schemes do not, however, appear to allow the simultaneous detection of multiple analytes on a single detector platform.

The alternative to antigen detection is the detection of nucleic acids. An approach for diagnostic testing with nucleic acids uses hybridization to target unique regions of the target organism. These techniques require fewer organisms ( $10^3$  to  $10^5$ ), but require about five hours to complete. As with antibody-antigen reactions this approach has not been developed for the simultaneous detection of multiple analytes.

The most recent improvement in the detection of microorganisms has been the use of nucleic acid amplification. Nucleic acid amplification tests have been developed that generate both qualitative and quantitative data. However, the current limitations of these testing methods are related to delays caused by specimen preparation, amplification, and detection. Currently, the standard assays require about five hours to complete. The ability to complete much faster detection for a variety of microorganisms would be of tremendous importance to military intelligence, national safety, medical, environmental, and food areas.

It is therefore desirable that new sensors capable of discriminating different analytes, toxins, and bacteria be developed for medical/clinical diagnostic, environmental, health and safety, remote sensing, military, food/beverage, and chemical processing applications. It is further desired that the sensing system be adaptable to

the simultaneous detection of a variety of analytes to improve throughput during various chemical and biological analytical procedures.

### SUMMARY OF THE INVENTION

5 Herein we describe a system and method for the analysis of a fluid containing one or more analytes. The system may be used for either liquid or gaseous fluids. The system, in some embodiments, may generate patterns that are diagnostic for both the individual analytes and mixtures of the analytes. The system in some embodiments, is made of a plurality of chemically sensitive particles, formed in an ordered array, capable of simultaneously detecting many different kinds of analytes rapidly. An aspect of the system is that the array may be formed using a  
10 microfabrication process, thus allowing the system to be manufactured in an inexpensive manner.

In an embodiment of a system for detecting analytes, the system, in some embodiments, includes a light source, a sensor array, and a detector. The sensor array, in some embodiments, is formed of a supporting member which is configured to hold a variety of chemically sensitive particles (herein referred to as "particles") in an ordered array. The particles are, in some embodiments, elements which will create a detectable signal in the presence of an  
15 analyte. The particles may produce optical (e.g., absorbance or reflectance) or fluorescence/phosphorescent signals upon exposure to an analyte. Examples of particles include, but are not limited to functionalized polymeric beads, agarous beads, dextrose beads, polyacrylamide beads, control pore glass beads, metal oxides particles (e.g., silicon dioxide ( $\text{SiO}_2$ ) or aluminum oxides ( $\text{Al}_2\text{O}_3$ )), polymer thin films, metal quantum particles (e.g., silver, gold, platinum, etc.), and semiconductor quantum particles (e.g., Si, Ge, GaAs, etc.). A detector (e.g., a charge-coupled  
20 device "CCD") in one embodiment is positioned below the sensor array to allow for the data acquisition. In another embodiment, the detector may be positioned above the sensor array to allow for data acquisition from reflectance of the light off of the particles.

Light originating from the light source may pass through the sensor array and out through the bottom side of the sensor array. Light modulated by the particles may pass through the sensor array and onto the proximally  
25 spaced detector. Evaluation of the optical changes may be completed by visual inspection or by use of a CCD detector by itself or in combination with an optical microscope. A microprocessor may be coupled to the CCD detector or the microscope. A fluid delivery system may be coupled to the supporting member of the sensor array. The fluid delivery system, in some embodiments, is configured to introduce samples into and out of the sensor array.

30 In an embodiment, the sensor array system includes an array of particles. The particles may include a receptor molecule coupled to a polymeric bead. The receptors, in some embodiments, are chosen for interacting with analytes. This interaction may take the form of a binding/association of the receptors with the analytes. The supporting member may be made of any material capable of supporting the particles, while allowing the passage of the appropriate wavelengths of light. The supporting member may include a plurality of cavities. The cavities may  
35 be formed such that at least one particle is substantially contained within the cavity.

In an embodiment, the optical detector may be integrated within the bottom of the supporting member, rather than using a separate detecting device. The optical detectors may be coupled to a microprocessor to allow evaluation of fluids without the use of separate detecting components. Additionally, a fluid delivery system may

also be incorporated into the supporting member. Integration of detectors and a fluid delivery system into the supporting member may allow the formation of a compact and portable analyte sensing system.

A high sensitivity CCD array may be used to measure changes in optical characteristics which occur upon binding of the biological/chemical agents. The CCD arrays may be interfaced with filters, light sources, fluid delivery and micromachined particle receptacles, so as to create a functional sensor array. Data acquisition and handling may be performed with existing CCD technology. CCD detectors may be configured to measure white light, ultraviolet light or fluorescence. Other detectors such as photomultiplier tubes, charge induction devices, photo diodes, photodiode arrays, and microchannel plates may also be used.

A particle, in some embodiments, possess both the ability to bind the analyte of interest and to create a modulated signal. The particle may include receptor molecules which possess the ability to bind the analyte of interest and to create a modulated signal. Alternatively, the particle may include receptor molecules and indicators. The receptor molecule may possess the ability to bind to an analyte of interest. Upon binding the analyte of interest, the receptor molecule may cause the indicator molecule to produce the modulated signal. The receptor molecules may be naturally occurring or synthetic receptors formed by rational design or combinatorial methods. Some examples of natural receptors include, but are not limited to, DNA, RNA, proteins, enzymes, oligopeptides, antigens, and antibodies. Either natural or synthetic receptors may be chosen for their ability to bind to the analyte molecules in a specific manner.

In one embodiment, a naturally occurring or synthetic receptor is bound to a polymeric bead in order to create the particle. The particle, in some embodiments, is capable of both binding the analyte(s) of interest and creating a detectable signal. In some embodiments, the particle will create an optical signal when bound to an analyte of interest.

A variety of natural and synthetic receptors may be used. The synthetic receptors may come from a variety of classes including, but not limited to, polynucleotides (e.g., aptamers), peptides (e.g., enzymes and antibodies), synthetic receptors, polymeric unnatural biopolymers (e.g., polythioureas, polyguanidiniums), and imprinted polymers. Polynucleotides are relatively small fragments of DNA which may be derived by sequentially building the DNA sequence. Peptides include natural peptides such as antibodies or enzymes or may be synthesized from amino acids. Unnatural biopolymers are chemical structure which are based on natural biopolymers, but which are built from unnatural linking units. For example, polythioureas and polyguanidiniums have a structure similar to peptides, but may be synthesized from diamines (i.e., compounds which include at least two amine functional groups) rather than amino acids. Synthetic receptors are designed organic or inorganic structures capable of binding various analytes.

In an embodiment, a large number of chemical/biological agents of interest to the military and civilian communities may be sensed readily by the described array sensors. Bacteria may also be detected using a similar system. To detect, sense, and identify intact bacteria, the cell surface of one bacteria may be differentiated from other bacteria, or genomic material may be detected using oligonucleic receptors. One method of accomplishing this differentiation is to target cell surface oligosaccharides (i.e., sugar residues). The use of synthetic receptors which are specific for oligosaccharides may be used to determine the presence of specific bacteria by analyzing for cell surface oligosaccharides.



### BRIEF DESCRIPTION OF THE DRAWINGS

The above brief description as well as further objects, features and advantages of the methods and apparatus of the present invention will be more fully appreciated by reference to the following detailed description of presently preferred but nonetheless illustrative embodiments in accordance with the present invention when taken in conjunction with the accompanying drawings in which:

FIG. 1 depicts a schematic of an analyte detection system;

FIG. 2 depicts a particle disposed in a cavity;

FIG. 3 depicts a sensor array;

FIG. 4A-F depicts the formation of a Fabry-Perot cavity on the back of a sensor array;

FIG. 5 depicts the chemical constituents of a particle;

FIG. 6 depicts the chemical formulas of some receptor compounds;

FIG. 7 depicts a plot of the absorbance of green light vs. concentration of calcium ( $\text{Ca}^{+2}$ ) for a particle which includes an *o*-cresolphthalein complexone receptor;

FIG. 8 depicts a schematic view of the transfer of energy from a first indicator to a second indicator in the presence of an analyte;

FIG. 9 depicts a schematic of the interaction of a sugar molecule with a boronic acid based receptor.

FIG. 10 depicts various synthetic receptors;

FIG. 11 depicts a synthetic pathway for the synthesis of polythioureas;

FIG. 12 depicts a synthetic pathway for the synthesis of polyguanidiniums;

FIG. 13 depicts a synthetic pathway for the synthesis of diamines from amino acids;

FIG. 14 depicts fluorescent diamino monomers;

FIG. 15 depicts a plot of counts/sec. (i.e., intensity) vs. time as the pH of a solution surrounding a particle coupled to *o*-cresolphthalein is cycled from acidic to basic conditions;

FIG. 16 depicts the color responses of a variety of sensing particles to solutions of  $\text{Ca}^{+2}$  and various pH levels;

FIG. 17 depicts an analyte detection system which includes a sensor array disposed within a chamber;

FIG. 18 depicts an integrated analyte detection system;

FIG. 19 depicts a cross-sectional view of a cavity covered by a mesh cover;

FIG. 20 depicts a top view of a cavity covered by a mesh cover;

FIG. 21A-G depicts a cross-sectional view of a series of processing steps for the formation of a sensor array which includes a removable top and bottom cover;

FIG. 22A-G depicts a cross-sectional view of a series of processing steps for the formation of a sensor array which includes a removable top and a stationary bottom cover;

FIG. 23A-G depicts a cross-sectional view of a series of processing steps for the formation of a sensor array which includes a removable top;

FIG. 24A-D depicts a cross-sectional view of a series of processing steps for the formation of a silicon based sensor array which includes a top and bottom cover with openings aligned with the cavity;

FIG. 25A-D depicts a cross-sectional view of a series of processing steps for the formation of a photoresist based sensor array which includes a top and bottom cover with openings aligned with the cavity;

FIG. 26A-E depicts a cross-sectional view of a series of processing steps for the formation of a plastic based sensor array which includes a top and bottom cover with openings aligned with the cavity;

FIG. 27A-D depicts a cross-sectional view of a series of processing steps for the formation of a silicon based sensor array which includes a top cover with openings aligned with the cavity and a tapered cavity;

FIG. 28A-E depicts a cross-sectional view of a series of processing steps for the formation of a photoresist based sensor array which includes a top cover with openings aligned with the cavity and a tapered cavity;

FIG. 29A-E depicts a cross-sectional view of a series of processing steps for the formation of a photoresist based sensor array which includes a top cover with openings aligned with the cavity and a bottom cover;

FIG. 30A-D depicts a cross-sectional view of a series of processing steps for the formation of a plastic based sensor array which includes a top cover with openings aligned with the cavity and a bottom cover;

FIG. 31 depicts a cross-sectional view of a schematic of a micropump;

FIG. 32 depicts a top view of an electrohydrodynamic pump;

FIG. 33 depicts a cross-sectional view of a sensor array which includes a micropump;

FIG. 34 depicts a cross-sectional view of a sensor array which includes a micropump and channels which are coupled to the cavities;

FIG. 35 depicts a cross-sectional view of a sensor array which includes multiple micropumps each micropump being coupled to a cavity;

FIG. 36 depicts a top view of a sensor array which includes multiple electrohydrodynamic pumps;

FIG. 37 depicts a cross-sectional view of a sensor array which includes a system for delivering a reagent from a reagent particle to a sensing cavity.

### **DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS**

Herein we describe a system and method for the simultaneous analysis of a fluid containing multiple analytes. The system may be used for either liquid or gaseous fluids. The system may generate patterns that are diagnostic for both individual analytes and mixtures of the analytes. The system, in some embodiments, is made of a combination of chemically sensitive particles, formed in an ordered array, capable of simultaneously detecting many different kinds of analytes rapidly. An aspect of the system is that the array may be formed using a microfabrication process, thus allowing the system to be manufactured in an inexpensive manner.

### **SYSTEM FOR ANALYSIS OF ANALYTES**

Shown in FIG. 1 is an embodiment of a system for detecting analytes in a fluid. The system, in some embodiments, includes a light source 110, a sensor array 120 and a detector 130. The light source 110 may be a white light source or light emitting diodes (LED). In one embodiment, light source 110 may be a blue light emitting diode (LED) for use in systems relying on changes in fluorescence signals. For colorimetric (e.g., absorbance) based systems, a white light source may be used. The sensor array 120, in some embodiments, is formed of a supporting member which is configured to hold a variety of particles 124. A detecting device 130 (e.g., a charge-coupled device "CCD") may be positioned below the sensor array to allow for data acquisition. In another embodiment, the detecting device 130 may be positioned above the sensor array.

Light originating from the light source 110, in some embodiments, passes through the sensor array 120 and out through the bottom side of the sensor array. The supporting member and the particles together, in some embodiments, provide an assembly whose optical properties are well matched for spectral analyses. Thus, light modulated by the particles may pass through the sensor array and onto the proximally spaced detector 130.

5 Evaluation of the optical changes may be completed by visual inspection (e.g., with a microscope) or by use of a microprocessor 140 coupled to the detector. For fluorescence measurements, a filter 135 may be placed between supporting member 120 and detector 130 to remove the excitation wavelength. A fluid delivery system 160 may be coupled to the supporting member. The fluid delivery system 160 may be configured to introduce samples into and out of the sensor array.

10 In an embodiment, the sensor array system includes an array of particles. Upon the surface and within the interior region of the particles are, in some embodiments, located a variety of receptors for interacting with analytes. The supporting member, in some embodiments, is used to localize these particles as well as to serve as a microenvironment in which the chemical assays can be performed. For the chemical/biological agent sensor arrays, the particles used for analysis are about 0.05 - 500 microns in diameter, and may actually change size (e.g., swell or  
15 shrink) when the chemical environment changes. Typically, these changes occur when the array system is exposed to the fluid stream which includes the analytes. For example, a fluid stream which comprises a non-polar solvent, may cause non-polar particles to change in volume when the particles are exposed to the solvent. To accommodate these changes, it is preferred that the supporting member consist of an array of cavities which serve as micro test-tubes.

20 The supporting member may be made of any material capable of supporting the particles, while allowing the passage of the appropriate wavelength of light. The supporting member is also made of a material substantially impervious to the fluid in which the analyte is present. A variety of materials may be used including plastics, glass, silicon based materials (e.g., silicon, silicon dioxide, silicon nitride, etc.) and metals. In one embodiment, the supporting member includes a plurality of cavities. The cavities may be formed such that at least one particle is  
25 substantially contained within the cavity. Alternatively, a plurality of particles may be contained within a single cavity.

In an embodiment, the supporting member may consist of a strip of plastic which is substantially transparent to the wavelength of light necessary for detection. A series of cavities may be formed within the strip. The cavities may be configured to hold at least one particle. The particles may be contained within the strip by a  
30 transparent cover which is configured to allow passage of the analyte containing fluid into the cavities.

In another embodiment, the supporting member may be formed using a silicon wafer as depicted in FIG. 2. The silicon wafer 210 may include a substantially transparent layer 220 formed on the bottom surface of the wafer. The cavities 230, in one embodiment, are formed by an anisotropic etch process of the silicon wafer. In one embodiment, anisotropic etching of the silicon wafer is accomplished using a wet hydroxide etch.

35 Photolithographic techniques may be used to define the locations of the cavities. The cavities may be formed such that the sidewalls of the cavities are substantially tapered at an angle of between about 50 to 60 degrees. Formation of such angled cavities may be accomplished by wet anisotropic etching of <100> silicon. The term "<100> silicon" refers to the crystal orientation of the silicon wafer. Other types of silicon, (e.g., <110> and <111> silicon) may lead to steeper angled sidewalls. For example, <111> silicon may lead to sidewalls formed at about 90

degrees. The angled sides of the cavities in some embodiments, serve as "mirror layers" which may improve the light collection efficiency of the cavities. The etch process may be controlled so that the formed cavities extend through the silicon wafer to the upper surface of transparent layer 220. While depicted as pyramidal, the cavities may be formed in a number of shapes including but not limited to, spherical, oval, cubic, or rectangular. An advantage to using a silicon wafer for the support member, is that the silicon material is substantially opaque to the light produced from the light source. Thus, the light may be inhibited from passing from one cavity to adjacent cavities. In this manner, light from one cavity may be inhibited from influencing the spectroscopic changes produced in an adjacent cavity.

The silicon wafer, in some embodiments, has an area of approximately  $1\text{ cm}^2$  to about  $100\text{ cm}^2$  and includes about  $10^1$  to about  $10^6$  cavities. In an embodiment, about 100 cavities are formed in a ten by ten matrix. The center to center distance between the cavities, in some embodiments, is about 500 microns. Each of the cavities may include at least one particle.

The transparent layer 220 may serve as a window, allowing light of a variety of wavelengths to pass through the cavities 230 and to the detector. Additionally, the transparent layer may serve as a platform onto which the individual particles 235 may be positioned. The transparent layer may be formed of silicon dioxide ( $\text{SiO}_2$ ), silicon nitride ( $\text{Si}_3\text{N}_4$ ) or silicon dioxide/silicon nitride multi-layer stacks. The transparent layer, in some embodiments, is deposited onto the silicon wafer prior to the formation of the cavities.

The cavities 230 may be sized to substantially contain a particle 235. The cavities are, in some embodiments, larger than a particle. The cavities are, in some embodiments, sized to allow facile placement and removal of the particle within the cavities. The cavity may be substantially larger than the particle, thus allowing the particle to swell during use. For example, a particle may have a size as depicted in FIG. 2 by particle 235. During use, contact with a fluid (e.g., a solvent) may cause the particle to swell, for example, to a size depicted as circle 236. In some embodiments, the cavity is sized to allow such swelling of the particle during use. A particle may be positioned at the bottom of a cavity using, e.g., a micromanipulator. After a particle has been placed within the cavity, a transparent cover plate 240 may be placed on top of the supporting member to keep the particle in place.

When forming an array which includes a plurality of particles, the particles may be placed in the array in an ordered fashion using the micromanipulator. In this manner, a ordered array having a predefined configuration of particles may be formed. Alternatively, the particles may be randomly placed within the cavities. The array may subsequently undergo a calibration test to determine the identity of the particle at any specified location in the supporting member.

The transparent cover plate 240, in some embodiments, is coupled to the upper surface of the silicon wafer 220 such that the particles are inhibited from becoming dislodged from the cavity. The transparent cover plate, in some embodiments, is positioned a fixed distance above the silicon wafer, as depicted in FIG. 2, to keep the particle in place, while allowing the entrance of fluids into the cavities. The transparent cover plate, in some embodiments, is positioned at a distance above the substrate which is substantially less than a width of the particle. The transparent cover plate may be made of any material which is substantially transparent to the wavelength of light being utilized by the detector. The transparent cover plate may be made of plastic, glass, quartz, or silicon dioxide/silicon nitride.

In one embodiment, the transparent cover plate 240, is a thin sheet of glass (e.g., a microscope slide cover slip). The slide may be positioned a fixed distance above the silicon wafer. Support structures 241 (See FIG. 2) may be placed upon the silicon wafer 210 to position the transparent cover plate 240. The support structures may be formed from a polymer or a silicon based material. In another embodiment, a polymeric substrate is coupled to the silicon wafer to form the support structures 241 for the transparent cover plate 240. In an embodiment, a plastic material with an adhesive backing (e.g., cellophane tape) is positioned on the silicon wafer 210. After the support structures 241 are placed on the wafer the transparent cover plate 240 is placed upon the support structures. The support structures inhibit the transparent cover sheet from contacting the silicon wafer 200. In this manner, a channel is formed between the silicon wafer and the transparent cover plate which allow the fluid to pass into the cavity, while inhibiting displacement of the particle by the fluid.

In another embodiment, the transparent cover plate 240 may be fastened to the upper surface of the silicon wafer, as depicted in FIG. 3. In this embodiment, the fluid may be inhibited from entering the cavities 230 by the transparent cover plate 240. To allow passage of the fluid into the cavities, a number of channels 250 may be formed in the silicon wafer. The channels, in one embodiment, are oriented to allow passage of the fluid into substantially all of the cavities. When contacted with the fluid, the particles may swell to a size which may plug the channels. To prevent this plugging, the channels may be formed near the upper portion of the cavities, as depicted in FIG 3. The channels, in one embodiment, are formed using standard photolithographic masking to define the regions where the trenches are to be formed, followed by the use of standard etching techniques. A depth of the cavity may be such that the particle resides substantially below the position of the channel. In this way, the plugging of the channels due to swelling of the particle may be prevented.

The inner surfaces of the cavities may be coated with a material to aid the positioning of the particles within the cavities. In one embodiment, a thin layer of gold or silver may be used to line the inner surface of the cavities. The gold or silver layer may act as an anchoring surface to anchor particles (e.g., via alkylthiol bonding). In addition, the gold or silver layer may also increase the reflectivity of the inner surface of the cavities. The increased reflectance of the surface may enhance the analyte detection sensitivity of the system. Alternatively, polymer layers and self-assembled monolayers formed upon the inner surface of the cavities may be used to control the particle adhesion interactions. Additional chemical anchoring methods may be used for silicon surfaces such as those based on siloxane type reagents, which may be attached to Si-OH functionalities. Similarly, monomeric and polymeric reagents attached to an interior region of the cavities can be used to alter the local wetting characteristics of the cavities. This type of methodology can be used to anchor the particles as well as to alter the fluid delivery characteristics of the cavity. Furthermore, amplification of the signals for the analytes may be accomplished with this type of strategy by causing preconcentration of appropriate analytes in the appropriate type of chemical environment.

In another embodiment, the optical detector may be integrated within the bottom transparent layer 220 of the supporting member, rather than using a separate detecting device. The optical detectors may be formed using a semiconductor-based photodetector 255. The optical detectors may be coupled to a microprocessor to allow evaluation of fluids without the use of separate detecting components. Additionally, the fluid delivery system may also be incorporated into the supporting member. Micro-pumps and micro-valves may also be incorporated into the silicon wafer to aid passage of the fluid through the cavities. Integration of detectors and a fluid delivery system

into the supporting member may allow the formation of a compact and portable analyte sensing system. Optical filters may also be integrated into the bottom membrane of the cavities. These filters may prevent short wavelength excitation from producing "false" signals in the optical detection system (e.g., a CCD detector array) during fluorescence measurements.

5 A sensing cavity may be formed on the bottom surface of the support substrate. An example of a sensing cavity that may be used is a Fabry-Perot type cavity. Fabry-Perot cavity-based sensors may be used to detect changes in optical path length induced by either a change in the refractive index or a change in physical length of the cavity. Using micromachining techniques, Fabry-Perot sensors may be formed on the bottom surface of the cavity.

10 Figures 4A-F depict a sequence of processing steps for the formation of a cavity and a planar top diaphragm Fabry-Perot sensor on the bottom surface of a silicon based supporting member. A sacrificial barrier layer 262a/b is deposited upon both sides of a silicon supporting member 260. The silicon supporting member 260 may be a double-side polished silicon wafer having a thickness ranging from about 100  $\mu\text{m}$  to about 500  $\mu\text{m}$ , preferably from about 200  $\mu\text{m}$  to about 400  $\mu\text{m}$ , and more preferably of about 300  $\mu\text{m}$ . The barrier layer 262a/b  
15 may be composed of silicon dioxide, silicon nitride, or silicon oxynitride. In one embodiment, the barrier layer 262a/b is composed of a stack of dielectric materials. As depicted in FIG 4A, the barrier layer 262 a/b is composed of a stack of dielectric materials which includes a silicon nitride layer 271a/b and a silicon dioxide layer 272a/b. Both layers may be deposited using a low pressure chemical vapor deposition ("LPCVD") process. Silicon nitride may be deposited using an LPCVD reactor by reaction of ammonia ( $\text{NH}_3$ ) and dichlorosilane ( $\text{SiCl}_2\text{H}_2$ ) at a gas  
20 flow rate of about 3.5:1, a temperature of about 800  $^\circ\text{C}$ , and a pressure of about 220 mTorr. The silicon nitride layer 271a/b is deposited to a thickness in the range from about 100  $\text{\AA}$  to about 500  $\text{\AA}$ , preferably from 200  $\text{\AA}$  to about 400  $\text{\AA}$ , and more preferably of about 300  $\text{\AA}$ . Silicon dioxide is may be deposited using an LPCVD reactor by reaction of silane ( $\text{SiH}_4$ ) and oxygen ( $\text{O}_2$ ) at a gas flow rate of about 3:4, a temperature of about 450  $^\circ\text{C}$ , and a  
25 pressure of about 110 mTorr. The silicon dioxide layer 272a/b is deposited to a thickness in the range from about 3000  $\text{\AA}$  to about 7000  $\text{\AA}$ , preferably from 4000  $\text{\AA}$  to about 6000  $\text{\AA}$ , and more preferably of about 5000  $\text{\AA}$ . The front face silicon dioxide layer 272a, in one embodiment, acts as the main barrier layer. The underlying silicon nitride layer 271a acts as an intermediate barrier layer to inhibit overetching of the main barrier layer during subsequent KOH wet anisotropic etching steps.

A bottom diaphragm layer 264a/b is deposited upon the barrier layer 262a/b on both sides of the  
30 supporting member 260. The bottom diaphragm layer 264a/b may be composed of silicon nitride, silicon dioxide, or silicon oxynitride. In one embodiment, the bottom diaphragm layer 264 a/b is composed of a stack of dielectric materials. As depicted in FIG 4A, the bottom diaphragm layer 264a/b is composed of a stack of dielectric materials which includes a pair of silicon nitride layers 273a/b and 275a/b surrounding a silicon dioxide layer 274a/b. All of the layers may be deposited using an LPCVD process. The silicon nitride layers 273a/b and 275a/b have a  
35 thickness in the range from about 500  $\text{\AA}$  to about 1000  $\text{\AA}$ , preferably from 700  $\text{\AA}$  to about 800  $\text{\AA}$ , and more preferably of about 750  $\text{\AA}$ . The silicon dioxide layer 274a/b has a thickness in the range from about 3000  $\text{\AA}$  to about 7000  $\text{\AA}$ , preferably from 4000  $\text{\AA}$  to about 6000  $\text{\AA}$ , and more preferably of about 4500  $\text{\AA}$ .

A cavity which will hold the particle may now be formed in the supporting member 260. The bottom diaphragm layer 264b and the barrier layer 262b formed on the back side 261 of the silicon supporting member 260

are patterned and etched using standard photolithographic techniques. In one embodiment, the layers are subjected to a plasma etch process. The plasma etching of silicon dioxide and silicon nitride may be performed using a mixture of carbontetrafluoride ( $\text{CF}_4$ ) and oxygen ( $\text{O}_2$ ). The patterned back side layers 262b and 264b may be used as a mask for anisotropic etching of the silicon supporting member 260. The silicon supporting member 260, in one embodiment, is anisotropically etched with a 40% potassium hydroxide ("KOH") solution at  $80^\circ\text{C}$  to form the cavity. The etch is stopped when the front side silicon nitride layer 271a is reached, as depicted in FIG 4B. The silicon nitride layer 271a inhibits etching of the main barrier layer 272a during this etch process. The cavity 267 may be formed extending through the supporting member 260. After formation of the cavity, the remaining portions of the back side barrier layer 262b and the diaphragm layer 264b may be removed.

Etch windows 266 are formed through the bottom diaphragm layer 264a on the front side of the wafer. A masking layer (not shown) is formed over the bottom diaphragm layer 264a and patterned using standard photolithographic techniques. Using the masking layer, etch windows 266 may be formed using a plasma etch. The plasma etching of silicon dioxide and silicon nitride may be performed using a mixture of carbontetrafluoride ( $\text{CF}_4$ ) and oxygen ( $\text{O}_2$ ). The etching is continued through the bottom diaphragm layer 264a and partially into the barrier layer 262a. In one embodiment, the etching is stopped at approximately half the thickness of the barrier layer 262a. Thus, when the barrier layer 262a is subsequently removed the etch windows 266 will extend through the bottom diaphragm layer 264a, communicating with the cavity 267. By stopping the etching at a midpoint of the barrier layer, voids or discontinuities may be reduced since the bottom diaphragm is still continuous due to the remaining barrier layer.

After the etch windows 266 are formed, a sacrificial spacer layer 268a/b is deposited upon the bottom diaphragm layer 264a and within cavity 267, as depicted in FIG. 4C. The spacer layer may be formed from LPCVD polysilicon. In one embodiment, the front side deposited spacer layer 268a will also at least partially fill the etch windows 266. Polysilicon may be deposited using an LPCVD reactor using silane ( $\text{SiH}_4$ ) at a temperature of about  $650^\circ\text{C}$ . The spacer layer 268a/b is deposited to a thickness in the range from about  $4000\text{ \AA}$  to about  $10,000\text{ \AA}$ , preferably from  $6000\text{ \AA}$  to about  $8000\text{ \AA}$ , and more preferably of about  $7000\text{ \AA}$ . The preferred thickness of the spacer layer 268a is dependent on the desired thickness of the internal air cavity of the Fabry-Perot detector. For example, if a Fabry-Perot detector which is to include a  $7000\text{ \AA}$  air cavity between the top and bottom diaphragm layer is desired, a spacer layer having a thickness of about  $7000\text{ \AA}$  would be formed. After the spacer layer has been deposited, a masking layer for etching the spacer layer 268a (not shown) is used to define the etch regions of the spacer layer 268a. The etching may be performed using a composition of nitric acid ( $\text{HNO}_3$ ), water, and hydrogen fluoride (HF) in a ratio of 25:13:1, respectively, by volume. The lateral size of the subsequently formed cavity is determined by the masking pattern used to define the etch regions of the spacer layer 268a.

After the spacer layer 268a has been etched, the top diaphragm layer 270a/b is formed. The top diaphragm 270a/b, in one embodiment, is deposited upon the spacer layer 268a/b on both sides of the supporting member. The top diaphragm 270a/b may be composed of silicon nitride, silicon dioxide, or silicon oxynitride. In one embodiment, the top diaphragm 270a/b is composed of a stack of dielectric materials. As depicted in FIG. 4C, the top diaphragm 270a/b is composed of a stack of dielectric materials which includes a pair of silicon nitride layers 283a/b and 285a/b surrounding a silicon dioxide layer 284a/b. All of the layers may be deposited using an LPCVD process. The silicon nitride layers 283a/b and 285a/b have a thickness in the range from about  $1000\text{ \AA}$  to about

2000 Å, preferably from 1200 Å to about 1700 Å, and more preferably of about 1500 Å. The silicon dioxide layer 284a/b has a thickness in the range from about 5000 Å to about 15,500 Å, preferably from 7500 Å to about 12,000 Å, and more preferably of about 10,500 Å.

After depositing the top diaphragm 270a/b, all of the layers stacked on the bottom face of the supporting member (e.g., layers 268b, 283b, 284b, and 285b) are removed by multiple wet and plasma etching steps, as depicted in FIG. 4D. After these layers are removed, the now exposed portions of the barrier layer 262a are also removed. This exposes the spacer layer 268a which is present in the etch windows 266. The spacer layer 268 may be removed from between the top diaphragm 270a and the bottom diaphragm 264a by a wet etch using a KOH solution, as depicted in FIG. 4D. Removal of the spacer material 268a, forms a cavity 286 between the top diaphragm layer 270a and the bottom diaphragm layer 264a. After removal of the spacer material, the cavity 286 may be washed using deionized water, followed by isopropyl alcohol to clean out any remaining etching solution.

The cavity 286 of the Fabry-Perot sensor may be filled with a sensing substrate 290, as depicted in FIG. 4E. To coat the cavity 286 with a sensing substrate 290, the sensing substrate may be dissolved in a solvent. A solution of the sensing substrate is applied to the supporting member 260. The solution is believed to rapidly enter the cavity 286 through the etched windows 266 in the bottom diaphragm 264a, aided in part by capillary action. As the solvent evaporates, a thin film of the sensing substrate 290 coats the inner walls of the cavity 286, as well as the outer surface of the bottom diaphragm 264a. By repeated treatment of the supporting member with the solution of the sensing substrate, the thickness of the sensing substrate may be varied.

In one embodiment, the sensing substrate 290 is poly(3-dodecylthiophene) whose optical properties change in response to changes in oxidation states. The sensing substrate poly(3-dodecylthiophene) may be dissolved in a solvent such as chloroform or xylene. In one embodiment, a concentration of about 0.1 g of poly(3-dodecylthiophene)/mL is used. Application of the solution of poly(3-dodecylthiophene) to the supporting member causes a thin film of poly(3-dodecylthiophene) to be formed on the inner surface of the cavity.

In some instances, the sensing substrate, when deposited within a cavity of a Fabry-Perot type detector, may cause stress in the top diaphragm of the detector. It is believed that when a sensing polymer coats a planar top diaphragm, extra residual stress on the top diaphragm causes the diaphragm to become deflected toward the bottom diaphragm. If the deflection becomes too severe, sticking between the top and bottom diaphragms may occur. In one embodiment, this stress may be relieved by the use of supporting members 292 formed within the cavity 286, as depicted in FIG. 4F. The supporting members 292 may be formed without any extra processing steps to the above described process flow. The formation of supporting members may be accomplished by deliberately leaving a portion of the spacer layer within the cavity. This may be accomplished by underetching the spacer layer (e.g., terminating the etch process before the entire etch process is finished). The remaining spacer will behave as a support member to reduce the deflection of the top diaphragm member. The size and shape of the support members may be adjusted by altering the etch time of the spacer layer, or adjusting the shape of the etch windows 266.

In another embodiment, a high sensitivity CCD array may be used to measure changes in optical characteristics which occur upon binding of the biological/chemical agents. The CCD arrays may be interfaced with filters, light sources, fluid delivery and micromachined particle receptacles, so as to create a functional sensor array. Data acquisition and handling may be performed with existing CCD technology. Data streams (e.g., red, green, blue for colorimetric assays; gray intensity for fluorescence assays) may be transferred from the CCD to a



computer via a data acquisition board. Current CCDs may allow for read-out rates of  $10^5$  pixels per second. Thus, the entire array of particles may be evaluated hundreds of times per second allowing for studies of the dynamics of the various host-guest interaction rates as well as the analyte/polymer diffusional characteristics. Evaluation of this data may offer a method of identifying and quantifying the chemical/biological composition of the test samples.

5 CCD detectors may be configured to measure white light, ultraviolet light or fluorescence. Other detectors such as photomultiplier tubes, charge induction devices, photodiode, photodiode arrays, and microchannel plates may also be used. It should be understood that while the detector is depicted as being positioned under the supporting member, the detector may also be positioned above the supporting member. It should also be understood that the detector typically includes a sensing element for detecting the spectroscopic events and a component for displaying  
10 the detected events. The display component may be physically separated from the sensing element. The sensing element may be positioned above or below the sensor array while the display component is positioned close to a user.

In one embodiment, a CCD detector may be used to record color changes of the chemical sensitive particles during analysis. As depicted in FIG. 1, a CCD detector 130 may be placed beneath the supporting  
15 member 120. The light transmitted through the cavities is captured and analyzed by the CCD detector. In one embodiment, the light is broken down into three color components, red, green and blue. To simplify the data, each color is recorded using 8 bits of data. Thus, the data for each of the colors will appear as a value between 0 and 255. The color of each chemical sensitive element may be represented as a red, blue and green value. For example, a blank particle (i.e., a particle which does not include a receptor) will typically appear white. For  
20 example, when broken down into the red, green and blue components, it is found that a typical blank particle exhibits a red value of about 253, a green value of about 250, and a blue value of about 222. This signifies that a blank particle does not significantly absorb red, green or blue light. When a particle with a receptor is scanned, the particle may exhibit a color change, due to absorbance by the receptor. For example, it was found that when a particle which includes a 5-carboxyfluorescein receptor is subjected to white light, the particle shows a strong  
25 absorbance of blue light. The CCD detector values for the 5-carboxyfluorescein particle exhibits a red value of about 254, a green value of about 218, and a blue value of about 57. The decrease in transmittance of blue light is believed to be due to the absorbance of blue light by the 5-carboxyfluorescein. In this manner, the color changes of a particle may be quantitatively characterized. An advantage of using a CCD detector to monitor the color changes is that color changes which may not be noticeable to the human eye may now be detected.

30 The support array may be configured to allow a variety of detection modes to be practiced. In one embodiment, a light source is used to generate light which is directed toward the particles. The particles may absorb a portion of the light as the light illuminates the particles. The light then reaches the detector, reduced in intensity by the absorbance of the particles. The detector may be configured to measure the reduction in light intensity (i.e., the absorbance) due to the particles. In another embodiment, the detector may be placed above the  
35 supporting member. The detector may be configured to measure the amount of light reflected off of the particles. The absorbance of light by the particles is manifested by a reduction in the amount of light being reflected from the cavity. The light source in either embodiment may be a white light source or a fluorescent light source.

## CHEMICALLY SENSITIVE PARTICLES

A particle, in some embodiments, possess both the ability to bind the analyte of interest and to create a modulated signal. The particle may include receptor molecules which possess the ability to bind the analyte of interest and to create a modulated signal. Alternatively, the particle may include receptor molecules and indicators.

5 The receptor molecule may possess the ability to bind to an analyte of interest. Upon binding the analyte of interest, the receptor molecule may cause the indicator molecule to produce the modulated signal. The receptor molecules may be naturally occurring or synthetic receptors formed by rational design or combinatorial methods. Some examples of natural receptors include, but are not limited to, DNA, RNA, proteins, enzymes, oligopeptides, antigens, and antibodies. Either natural or synthetic receptors may be chosen for their ability to bind to the analyte  
10 molecules in a specific manner. The forces which drive association/recognition between molecules include the hydrophobic effect, anion-cation attraction, and hydrogen bonding. The relative strengths of these forces depend upon factors such as the solvent dielectric properties, the shape of the host molecule, and how it complements the guest. Upon host-guest association, attractive interactions occur and the molecules stick together. The most widely used analogy for this chemical interaction is that of a "lock and key". The fit of the key molecule (the guest) into  
15 the lock (the host) is a molecular recognition event.

A naturally occurring or synthetic receptor may be bound to a polymeric resin in order to create the particle. The polymeric resin may be made from a variety of polymers including, but not limited to, agarous, dextrose, acrylamide, control pore glass beads, polystyrene-polyethylene glycol resin, polystyrene-divinyl benzene resin, formylpolystyrene resin, trityl-polystyrene resin, acetyl polystyrene resin, chloroacetyl polystyrene resin,  
20 aminomethyl polystyrene-divinylbenzene resin, carboxypolystyrene resin, chloromethylated polystyrene-divinylbenzene resin, hydroxymethyl polystyrene-divinylbenzene resin, 2-chlorotrityl chloride polystyrene resin, 4-benzyloxy-2'4'-dimethoxybenzhydrol resin (Rink Acid resin), triphenyl methanol polystyrene resin, diphenylmethanol resin, benzhydrol resin, succinimidyl carbonate resin, p-nitrophenyl carbonate resin, imidazole carbonate resin, polyacrylamide resin, 4-sulfamylbenzoyl-4'-methylbenzhydramine-resin (Safety-catch resin), 2-amino-2-(2'-nitrophenyl) propionic acid-aminomethyl resin (ANP Resin), p-benzyloxybenzyl alcohol-  
25 divinylbenzene resin (Wang resin), p-methylbenzhydramine-divinylbenzene resin (MBHA resin), Fmoc-2,4-dimethoxy-4'-(carboxymethoxy)-benzhydramine linked to resin (Knorr resin), 4-(2',4'-Dimethoxyphenyl)-Fmoc-aminomethyl)-phenoxy resin (Rink resin), 4-hydroxymethyl-benzoyl-4'-methylbenzhydramine resin (HMBA-MBHA Resin), p-nitrobenzophenone oxime resin (Kaiser oxime resin), and amino-2,4-dimethoxy-4'-(carboxymethoxy)-benzhydramine handle linked to 2-chlorotrityl resin (Knorr-2-chlorotrityl resin). In one  
30 embodiment, the material used to form the polymeric resin is compatible with the solvent in which the analyte is dissolved. For example, polystyrene-divinyl benzene resin will swell within non-polar solvents, but does not significantly swell within polar solvents. Thus, polystyrene-divinyl benzene resin may be used for the analysis of analytes within non-polar solvents. Alternatively, polystyrene-polyethylene glycol resin will swell with polar  
35 solvents such as water. Polystyrene-polyethylene glycol resin may be useful for the analysis of aqueous fluids.

In one embodiment, a polystyrene-polyethylene glycol-divinyl benzene material is used to form the polymeric resin. The polystyrene-polyethylene glycol-divinyl benzene resin is formed from a mixture of polystyrene 375, divinyl benzene 380 and polystyrene-polyethylene glycol 385, see FIG. 5. The polyethylene glycol portion of the polystyrene-polyethylene glycol 385, in one embodiment, may be terminated with an amine.

The amine serves as a chemical handle to anchor both receptors and indicator dyes. Other chemical functional groups may be positioned at the terminal end of the polyethylene glycol to allow appropriate coupling of the polymeric resin to the receptor molecules or indicators.

The chemically sensitive particle, in one embodiment, is capable of both binding the analyte(s) of interest and creating a detectable signal. In one embodiment, the particle will create an optical signal when bound to an analyte of interest. The use of such a polymeric bound receptors offers advantages both in terms of cost and configurability. Instead of having to synthesize or attach a receptor directly to a supporting member, the polymeric bound receptors may be synthesized *en masse* and distributed to multiple different supporting members. This allows the cost of the sensor array, a major hurdle to the development of mass-produced environmental probes and medical diagnostics, to be reduced. Additionally, sensor arrays which incorporate polymeric bound receptors may be reconfigured much more quickly than array systems in which the receptor is attached directly to the supporting member. For example, if a new variant of a pathogen or a pathogen that contains a genetically engineered protein is a threat, then a new sensor array system may be readily created to detect these modified analytes by simply adding new sensor elements (e.g., polymeric bound receptors) to a previously formed supporting member.

In one embodiment, a receptor, which is sensitive to changes in the pH of a fluid sample is bound to a polymeric resin to create a particle. That is, the receptor is sensitive to the concentration of hydrogen cations ( $H^+$ ). The receptor in this case is typically sensitive to the concentration of  $H^+$  in a fluid solution. The analyte of interest may therefore be  $H^+$ . There are many types of molecules which undergo a color change when the pH of the fluid is changed. For example, many types of dyes undergo significant color changes as the pH of the fluid medium is altered. Examples of receptors which may be used to monitor the pH of a fluid sample include 5-carboxyfluorescein and alizarin complexone, depicted in FIG. 6. Each of these receptors undergoes significant color changes as the pH of the fluid is altered. 5-carboxyfluorescein undergoes a change from yellow to orange as the pH of the fluid is increased. Alizarin complexone undergoes two color changes, first from yellow to red, then from red to blue as the pH of the fluid increases. By monitoring the change in color caused by dyes attached to a polymeric particle, the pH of a solution may be qualitatively and, with the use of a detector (e.g., a CCD detector), quantitatively monitored.

In another embodiment, a receptor which is sensitive to presence of metal cations is bound to a polymeric particle to create a particle. The receptor in this case is typically sensitive to the concentration of one or more metal cations present in a fluid solution. In general, colored molecules which will bind cations may be used to determine the presence of a metal cation in a fluid solution. Examples of receptors which may be used to monitor the presence of cations in a fluid sample include alizarin complexone and *o*-cresolphthalein complexone, see FIG. 6. Each of these receptors undergoes significant color changes as the concentration of a specific metal ion in the fluid is altered. Alizarin complexone is particularly sensitive to lanthanum ions. In the absence of lanthanum, alizarin complexone will exhibit a yellow color. As the concentration of lanthanum is increased, alizarin complexone will change to a red color. *o*-Cresolphthalein complexone is particularly sensitive to calcium ions. In the absence of calcium, *o*-cresolphthalein complexone is colorless. As the concentration of calcium is increased, *o*-cresolphthalein complexone will change to a blue color. By monitoring the change in color of metal cation sensitive receptors attached to a polymeric particle, the presence of a specific metal ion may be qualitatively and, with the use of a detector (e.g., a CCD detector), quantitatively monitored.

Referring to FIG. 7, a graph of the absorbance of green light vs. concentration of calcium ( $\text{Ca}^{+2}$ ) is depicted for a particle which includes an *o*-cresolphthalein complexone receptor. As the concentration of calcium is increased, the absorbance of green light increases in a linear manner up to a concentration of about 0.0006 M. A concentration of 0.0006 M is the solubility limit of calcium in the fluid, thus no significant change in absorbance is noted after this point. The linear relationship between concentration and absorbance allows the concentration of calcium to be determined by measuring the absorbance of the fluid sample.

In one embodiment, a detectable signal may be caused by the altering of the physical properties of an indicator ligand bound to the receptor or the polymeric resin. In one embodiment, two different indicators are attached to a receptor or the polymeric resin. When an analyte is captured by the receptor, the physical distance between the two indicators may be altered such that a change in the spectroscopic properties of the indicators is produced. A variety of fluorescent and phosphorescent indicators may be used for this sensing scheme. This process, known as Forster energy transfer, is extremely sensitive to small changes in the distance between the indicator molecules.

For example, a first fluorescent indicator 320 (e.g., a fluorescein derivative) and a second fluorescent indicator 330 (e.g., a rhodamine derivative) may be attached to a receptor 300, as depicted in FIG. 8. When no analyte is present short wavelength excitation 310 may excite the first fluorescent indicator 320, which fluoresces as indicated by 312. The short wavelength excitation, however, may cause little or no fluorescence of the second fluorescent indicator 330. After binding of analyte 350 to the receptor, a structural change in the receptor molecule may bring the first and second fluorescent indicators closer to each other. This change in intermolecular distance may allow the excited first indicator 320 to transfer a portion of its fluorescent energy 325 to the second fluorescent indicator 330. This transfer in energy may be measured by either a drop in energy of the fluorescence of the first indicator molecule 320, or the detection of increased fluorescence 314 by the second indicator molecule 330.

Alternatively, the first and second fluorescent indicators may initially be positioned such that short wavelength excitation, may cause fluorescence of both the first and second fluorescent indicators, as described above. After binding of analyte 350 to the receptor, a structural change in the receptor molecule may cause the first and second fluorescent indicators to move further apart. This change in intermolecular distance may inhibit the transfer of fluorescent energy from the first indicator 320 to the second fluorescent indicator 330. This change in the transfer of energy may be measured by either a drop in energy of the fluorescence of the second indicator molecule 330, or the detection of increased fluorescence by the first indicator molecule 320.

In another embodiment, an indicator ligand may be preloaded onto the receptor. An analyte may then displace the indicator ligand to produce a change in the spectroscopic properties of the particles. In this case, the initial background absorbance is relatively large and decreases when the analyte is present. The indicator ligand, in one embodiment, has a variety of spectroscopic properties which may be measured. These spectroscopic properties include, but are not limited to, ultraviolet absorption, visible absorption, infrared absorption, fluorescence, and magnetic resonance. In one embodiment, the indicator is a dye having either a strong fluorescence, a strong ultraviolet absorption, a strong visible absorption, or a combination of these physical properties. Examples of indicators include, but are not limited to, carboxyfluorescein, ethidium bromide, 7-dimethylamino-4-methylcoumarin, 7-diethylamino-4-methylcoumarin, eosin, erythrosin, fluorescein, Oregon Green 488, pyrene, Rhodamine Red, tetramethylrhodamine, Texas Red, Methyl Violet, Crystal Violet, Ethyl Violet, Malachite green,

Methyl Green, Alizarin Red S, Methyl Red, Neutral Red, *o*-cresolsulfonephthalein, *o*-cresolphthalein, phenolphthalein, Acridine Orange, *B*-naphthol, coumarin, and *a*-naphthionic acid. When the indicator is mixed with the receptor, the receptor and indicator interact with each other such that the above mentioned spectroscopic properties of the indicator, as well as other spectroscopic properties may be altered. The nature of this interaction may be a binding interaction, wherein the indicator and receptor are attracted to each other with a sufficient force to allow the newly formed receptor-indicator complex to function as a single unit. The binding of the indicator and receptor to each other may take the form of a covalent bond, an ionic bond, a hydrogen bond, a van der Waals interaction, or a combination of these bonds.

The indicator may be chosen such that the binding strength of the indicator to the receptor is less than the binding strength of the analyte to the receptor. Thus, in the presence of an analyte, the binding of the indicator with the receptor may be disrupted, releasing the indicator from the receptor. When released, the physical properties of the indicator may be altered from those it exhibited when bound to the receptor. The indicator may revert back to its original structure, thus regaining its original physical properties. For example, if a fluorescent indicator is attached to a particle that includes a receptor, the fluorescence of the particle may be strong before treatment with an analyte containing fluid. When the analyte interacts with the particle, the fluorescent indicator may be released. Release of the indicator may cause a decrease in the fluorescence of the particle, since the particle now has less indicator molecules associated with it.

An example of this type of system is illustrated by the use of a boronic acid substituted resin 505 as a particle. Prior to testing, the boronic acid substituted resin 505 is treated with a sugar 510 which is tagged with an indicator (e.g., resorufin) as depicted in FIG. 9. The sugar 510 binds to the boronic acid receptor 500 imparting a color change to the boronic substituted resin 505 (yellow for the resorufin tagged sugar). When the boronic acid resin 505 is treated with a fluid sample which includes a sugar 520, the tagged sugar 510 may be displaced, causing a decrease in the amount of color produced by the boronic acid substituted resin 505. This decrease may be qualitatively or, with the use of a detector (e.g., a CCD detector), quantitatively monitored.

In another embodiment, a designed synthetic receptor may be used. In one embodiment, a polycarboxylic acid receptor may be attached to a polymeric resin. The polycarboxylic receptors are discussed in U.S. patent application serial no. 08/950,712 which is incorporated herein by reference.

In an embodiment, the analyte molecules in the fluid may be pretreated with an indicator ligand. Pretreatment may involve covalent attachment of an indicator ligand to the analyte molecule. After the indicator has been attached to the analyte, the fluid may be passed over the sensing particles. Interaction of the receptors on the sensing particles with the analytes may remove the analytes from the solution. Since the analytes include an indicator, the spectroscopic properties of the indicator may be passed onto the particle. By analyzing the physical properties of the sensing particles after passage of an analyte stream, the presence and concentration of an analyte may be determined.

For example, the analytes within a fluid may be derivatized with a fluorescent tag before introducing the stream to the particles. As analyte molecules are adsorbed by the particles, the fluorescence of the particles may increase. The presence of a fluorescent signal may be used to determine the presence of a specific analyte. Additionally, the strength of the fluorescence may be used to determine the amount of analyte within the stream.

## RECEPTORS

A variety of natural and synthetic receptors may be used. The synthetic receptors may come from a variety of classes including, but not limited to, polynucleotides (e.g., aptamers), peptides (e.g., enzymes and antibodies), synthetic receptors, polymeric unnatural biopolymers (e.g., polythioureas, polyguanidiniums), and imprinted polymers., some of which are generally depicted in FIG. 10. Natural based synthetic receptors include receptors which are structurally similar to naturally occurring molecules. Polynucleotides are relatively small fragments of DNA which may be derived by sequentially building the DNA sequence. Peptides may be synthesized from amino acids. Unnatural biopolymers are chemical structure which are based on natural biopolymers, but which are built from unnatural linking units. Unnatural biopolymers such as polythioureas and polyguanidiniums may be synthesized from diamines (i.e., compounds which include at least two amine functional groups). These molecules are structurally similar to naturally occurring receptors, (e.g., peptides). Some diamines may, in turn, be synthesized from amino acids. The use of amino acids as the building blocks for these compounds allow a wide variety of molecular recognition units to be devised. For example, the twenty natural amino acids have side chains that possess hydrophobic residues, cationic and anionic residues, as well as hydrogen bonding groups. These side chains may provide a good chemical match to bind a large number of targets, from small molecules to large oligosaccharides. Amino acid based peptides, polythioureas, and polyguanidiniums are depicted in FIG. 10.

Techniques for the building of DNA fragments and polypeptide fragments on a polymer particle are well known. Techniques for the immobilization of naturally occurring antibodies and enzymes on a polymeric resin are also well known. The synthesis of polythioureas upon a resin particle may be accomplished by the synthetic pathway depicted in FIG. 11. The procedure may begin by deprotection of the terminal tBoc protecting group on an amino acid coupled to a polymeric particle. Removal of the protecting group is followed by coupling of the rigid spacer 410 to the resulting amine 405 using diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole hydrate (HOBT). The spacer group may inhibit formation of a thiazolone by reaction of the first amino acids with subsequently formed thioureas. After the spacer group is coupled to the amino acid, another tBoc deprotection is performed to remove the spacer protecting group, giving the amine 415. At this point, monomer may be added incrementally to the growing chain, each time followed by a tBoc deprotection. The addition of a derivative of the diamine 420 (e.g., an isothiocyanate) to amine 415 gives the mono-thiourea 425. The addition of a second thiourea substituent is also depicted. After the addition of the desired number of monomers, a solution of benzyliothiocyanate or acetic anhydride may be added to cap any remaining amines on the growing oligomers. Between 1 to 20 thioureas groups may be formed to produce a synthetic polythiourea receptor.

The synthesis of polyguanidiniums may be accomplished as depicted in FIG. 12. In order to incorporate these guanidinium groups into the receptor, the coupling of a thiourea with a terminal amine in the presence of Mukaiyama's reagent may be utilized. The coupling of the first thiourea diamine 430 with an amino group of a polymeric particle gives the mono-guanidinium 434. Coupling of the resulting mono-guanidinium with a second thiourea diamine 436 gives a di-guanidinium 438. Further coupling may create a tri-guanidinium 440. Between 1 to 20 guanidinium groups may be formed to produce a synthetic polyguanidinium receptor.

The above described methods for making polythioureas and polyguanidiniums are based on the incorporation of diamines (i.e., molecules which include at least two amine functional groups) into the oligomeric

receptor. The method may be general for any compound having at least two amino groups. In one embodiment, the diamine may be derived from amino acids. A method for forming diamines from amino acids is shown in FIG. 13. Treatment of a protected amino acid 450 with borane-THF reduces the carboxylic acid portion of the amino acid to the primary alcohol 452. The primary alcohol is treated with phthalimide under Mitsunobu conditions (PPh<sub>3</sub>/DEAD). The resulting compound 454 is treated with aqueous methylamine to form the desired monoprotected diamine 456. The process may be accomplished such that the enantiomeric purity of the starting amino acid is maintained. Any natural or synthetic amino acid may be used in the above described method.

The three coupling strategies used to form the respective functional groups may be completely compatible with each other. The capability to mix linking groups (amides, thioureas, and guanidiniums) as well as the side chains (hydrophobic, cationic, anionic, and hydrogen bonding) may allow the creation of a diversity in the oligomers that is beyond the diversity of receptors typically found with natural biological receptors. Thus, we may produce ultra-sensitive and ultra-selective receptors which exhibit interactions for specific toxins, bacteria, and environmental chemicals. Additionally, these synthetic schemes may be used to build combinatorial libraries of particles for use in the sensor array.

In an embodiment, the indicator ligand may be incorporated into synthetic receptors during the synthesis of the receptors. The ligand may be incorporated into a monomeric unit, such as a diamine, that is used during the synthesis of the receptor. In this manner, the indicator may be covalently attached to the receptor in a controlled position. By placing the indicator within the receptor during the synthesis of the receptor, the positioning of the indicator ligand within the receptor may be controlled. This control may be difficult to achieve after synthesis of the receptor is completed.

In one embodiment, a fluorescent group may be incorporated into a diamine monomer for use in the synthetic sequences. Examples of monomeric units which may be used for the synthesis of a receptor are depicted in FIG. 14. The depicted monomers include fluorescent indicator groups. After synthesis, the interaction of the receptor with the analyte may induce changes in the spectroscopic properties of the molecule. Typically, hydrogen bonding or ionic substituents on the fluorescent monomer involved in analyte binding have the capacity to change the electron density and/or rigidity of the fluorescent ring system, thereby causing observable changes in the spectroscopic properties of the indicator. For fluorescent indicators such changes may be exhibited as changes in the fluorescence quantum yield, maximum excitation wavelength, and/or maximum emission wavelength. This approach does not require the dissociation of a preloaded fluorescent ligand, which may be limited in response time by  $k_{\text{off}}$ . While fluorescent ligands are shown here, it is to be understood that a variety of other ligand may be used including colorimetric ligands.

In another embodiment, two fluorescent monomers for signaling may be used for the synthesis of the receptor. For example, compound 470 (a derivative of fluorescein) and compound 475 (a derivative of rhodamine), depicted in FIG. 14, may both be incorporated into a synthetic receptor. Compound 470 contains a common colorimetric/fluorescent probe that will, in some embodiments, send out a modulated signal upon analyte binding. The modulation may be due to resonance energy transfer to compound 475. When an analyte binds to the receptor, structural changes in the receptor may alter the distance between monomeric units 470 and 475. It is well known that excitation of fluorescein can result in emission from rhodamine when these molecules are oriented correctly. The efficiency of resonance energy transfer from monomers 470 to 475 will depend strongly upon the presence of

analyte binding; thus, measurement of rhodamine fluorescence intensity (at a substantially longer wavelength than fluorescein fluorescence) may serve as an indicator of analyte binding. To greatly improve the likelihood of a modulatory fluorescein-rhodamine interaction, multiple rhodamine tags may be attached at different sites along a receptor molecule without substantially increasing background rhodamine fluorescence (only rhodamine very close to fluorescein will yield appreciable signal). This methodology may be applied to a number of alternate fluorescent pairs.

In an embodiment, a large number of chemical/biological agents of interest to the military and civilian communities may be sensed readily by the described array sensors including both small and medium size molecules. For example, it is known that nerve gases typically produce phosphate structures upon hydrolysis in water. The presence of molecules which contain phosphate functional groups may be detected using polyguanidiniums. Nerve gases which have contaminated water sources may be detected by the use of the polyguanidinium receptors described above.

In order to identify, sense, and quantitate the presence of various bacteria using the proposed micro-machined sensor, two strategies may be used. First, small molecule recognition and detection may be exploited. Since each bacteria possesses a unique and distinctive concentration of the various cellular molecules, such as DNA, proteins, metabolites, and sugars, the fingerprint (i.e., the concentration and types of DNA, proteins, metabolites, and sugars) of each organism is expected to be unique. Hence, the analytes obtained from whole bacteria or broken down bacteria may be used to determine the presence of specific bacteria. A series of receptors specific for DNA molecules, proteins, metabolites, and sugars may be incorporated into an array. A solution containing bacteria, or more preferably broken down bacteria, may be passed over the array of particles. The individual cellular components of the bacteria may interact in a different manner with each of the particles. This interaction will provide a pattern within the array which may be unique for the individual bacteria. In this manner, the presence of bacteria within a fluid may be determined.

In another embodiment, bacteria may be detected as whole entities, as found in ground water, aerosols, or blood. To detect, sense, and identify intact bacteria, the cell surface of one bacteria may be differentiated from other bacteria. One method of accomplishing this differentiation is to target cell surface oligosaccharides (i.e. sugar residues). Each bacterial class (gram negative, gram positive, etc.) displays a different oligosaccharide on their cell surfaces. The oligosaccharide, which is the code that is read by other cells giving an identification of the cell, is part of the cell-cell recognition and communication process. The use of synthetic receptors which are specific for oligosaccharides may be used to determine the presence of specific bacteria by analyzing for the cell surface oligosaccharides.

In another embodiment, the sensor array may be used to optimize which receptor molecules should be used for a specific analyte. An array of receptors may be placed within the cavities of the supporting member and a stream containing an analyte may be passed over the array. The reaction of each portion of the sensing array to the known analyte may be analyzed and the optimal receptor determined by determining which particle, and therefore which receptor, exhibits the strongest reaction toward the analyte. In this manner, a large number of potential receptors may be rapidly scanned. The optimal receptor may then be incorporated into a system used for the detection of the specific analyte in a mixture of analytes.



It should be emphasized that although some particles may be purposefully designed to bind to important species (biological agents, toxins, nerve gasses, etc.), most structures will possess nonspecific receptor groups. One of the advantages associated with the proposed sensor array is the capacity to standardize each array of particles via exposure to various analytes, followed by storage of the patterns which arise from interaction of the analytes with the particles. Therefore, there may not be a need to know the identity of the actual receptor on each particle. Only the characteristic pattern for each array of particles is important. In fact, for many applications it may be less time consuming to place the various particles into their respective holders without taking precautions to characterize the location associated with the specific particles. When used in this manner, each individual sensor array may require standardization for the type of analyte to be studied.

On-site calibration for new or unknown toxins may also be possible with this type of array. Upon complexation of an analyte, the local microenvironment of each indicator may change, resulting in a modulation of the light absorption and/or emission properties. The use of standard pattern recognition algorithms completed on a computer platform may serve as the intelligence factor for the analysis. The "fingerprint" like response evoked from the simultaneous interactions occurring at multiple sites within the substrate may be used to identify the species present in unknown samples.

The above described sensor array system offers a number of distinct advantages over existing technologies. One advantage is that "real time" detection of analytes may be performed. Another advantage is that the simultaneous detection of multiple analytes may be realized. Yet another advantage is that the sensor array system allows the use of synthetic reagents as well as biologically produced reagents. Synthetic reagents typically have superior sensitivity and specificity toward analytes when compared to the biological reagents. Yet another advantage is that the sensor array system may be readily modified by simply changing the particles which are placed within the sensor array. This interchangeability may also reduce production costs.

## EXAMPLES

### 1. The determination of pH using a chemically sensitive particle.

Shown in FIG. 15 is the magnitude of the optical signal transmitted through a single polymer particle derivatized with o-cresolphthalein. Here, a filter is used to focus the analysis on those wavelengths which the dye absorbs most strongly (i.e., about 550 nm). Data is provided for the particle as the pH is cycled between acid and basic environments. In acidic media (i.e., at times of 100-150 seconds and 180-210 seconds), the particle is clear and the system yields large signals (up to greater than 300,000 counts) at the optical detector. Between times of 0-100 and 150-180 seconds, the solution was made basic. Upon raising the pH (i.e., making the solution more basic), the particle turns purple in color and the transmitted green light is greatly diminished. Large signal reductions are recorded under such circumstances. The evolution of the signal changes show that the response time is quite rapid, on the order of 10 seconds. Furthermore, the behavior is highly reproducible.

### 2. The simultaneous detection of $\text{Ca}^{+2}$ , $\text{Ce}^{+3}$ , and pH by a sensor array system.

The synthesis of four different particles was accomplished by coupling a variety of indicator ligands to a polyethylene glycol-polystyrene ("PEG-PS") resin particle. The PEG-PS resin particles were obtained from Novabiochem Corp., La Jolla, Ca. The particles have an average diameter of about 130  $\mu\text{m}$  when dry and about

250  $\mu\text{m}$  when wet. The indicator ligands of fluorescein, *o*-cresolphthalein complexone, and alizarin complexone were each attached to PEG-PS resin particles using a dicyclohexylcarbodiimide (DCC) coupling between a terminal resin bound amine and a carboxylic acid on the indicator ligand.

These synthetic receptors, localized on the PEG-PS resin to create sensing particles, were positioned within micromachined wells formed in silicon/silicon nitride wafers, thus confining the particles to individually addressable positions on a multicomponent chip. These wells were sized to hold the particles in both swollen and unswollen states. Rapid introduction of the test fluids can be accomplished using these structures while allowing spectrophotometric assays to probe for the presence of analytes. For the identification and quantification of analyte species, changes in the light absorption and light emission properties of the immobilized resin particles can be exploited, although only identification based upon absorption properties are discussed here. Upon exposure to analytes, color changes for the particles were found to be 90% complete within one minute of exposure, although typically only seconds were required. To make the analysis of the colorimetric changes efficient, rapid, and sensitive, a charge-coupled-device (CCD) was directly interfaced with the sensor array. Thus, data streams composed of red, green, and blue (RGB) light intensities were acquired and processed for each of the individual particle elements. The red, blue, and green responses of the particles to various solutions are graphically depicted in FIG. 16.

The true power of the described bead sensor array occurs when simultaneous evaluation of multiple chemically distinct bead structures is completed. A demonstration of the capacity of five different beads is provided in FIG. 16. In this case, blank, alizarin, *o*-cresolphthalein, fluorescein, and alizarin- $\text{Ce}^{3+}$  complex derivatized beads serve as a matrix for subtle differentiation of chemical environments. The blank bead is simply a polystyrene sphere with no chemical derivatization. The bead derivatized with *o*-cresolphthalein responds to  $\text{Ca}^{+2}$  at pHs values around 10.0. The binding of calcium is noted from the large green color attenuation noted for this dye while exposed to the cation. Similarly, the fluorescein derivatized bead acts as a pH sensor. At pHs below 7.4 it is light yellow, but at higher pHs it turns dark orange. Interestingly, the alizarin complexone plays three distinct roles. First, it acts as a proton sensor yielding a yellow color at pHs below 4.5, orange is noted at pHs between 4.5 and 11.5, and at pHs above 11.5 a blue hue is observed. Second, it functions as a sensor for lanthanum ions at lower pHs by turning yellow to orange. Third, the combination of both fluoride and lanthanum ions results in yellow/orange coloration.

The analysis of solutions containing various amount of  $\text{Ca}^{+2}$  or  $\text{F}^-$  at various pH levels was performed using alizarin complexone, *o*-cresolphthalein complexone, 5-carboxy fluorescein, and alizarin- $\text{Ce}^{3+}$  complex. A blank particle in which the terminal amines of a PEG-PS resin particle have been acylated was also used. In this example, the presence of  $\text{Ca}^{+2}$  (0.1 M  $\text{Ca}(\text{NO}_3)_2$ ) was analyzed under conditions of varying pH. The pH was varied to values of 2, 7, and 12, all buffered by a mixture of 0.04 M phosphate, 0.04 M acetate, and 0.04 M borate. The RGB patterns for each sensor element in all environments were measured. The bead derivatized with *o*-cresolphthalein responds to  $\text{Ca}^{+2}$  at pH values around 12. Similarly, the 5-carboxy fluorescein derivatized bead acts as a pH sensor. At pHs below 7.4 it is light yellow, but at higher pHs it turns dark orange. Interestingly, the alizarin complexone plays three distinct roles. First, it acts as a proton sensor yielding a yellow color at pHs below 4.5, orange is noted at pHs between 4.5 and 11.5, and at pHs above 11.5 a blue hue is observed. Second, it functions as

a sensor for lanthanum ions at lower pHs by turning yellow to orange. Third, the combination of both fluoride and lanthanum ions results in yellow/orange coloration.

This example demonstrates a number of important factors related to the design, testing, and functionality of micromachined array sensors for solution analyses. First, derivatization of polymer particles with both colorimetric and fluorescent dyes was completed. These structures were shown to respond to pH and  $\text{Ca}^{2+}$ . Second, response times well under 1 minute were found. Third, micromachined arrays suitable both for confinement of particles, as well as optical characterization of the particles, have been prepared. Fourth, integration of the test bed arrays with commercially available CCD detectors has been accomplished. Finally, simultaneous detection of several analytes in a mixture was made possible by analysis of the RGB color patterns created by the sensor array.

### 3. The detection of sugar molecules using a boronic acid based receptor.

A series of receptors were prepared with functionalities that associate strongly with sugar molecules, as depicted in FIG. 9. In this case, a boronic acid sugar receptor 500 was utilized to demonstrate the functionality of a new type of sensing scheme in which competitive displacement of a resorufin derivatized galactose sugar molecule was used to assess the presence (or lack thereof) of other sugar molecules. The boronic acid receptor 500 was formed via a substitution reaction of a benzylic bromide. The boronic acid receptor was attached to a polyethylene glycol-polystyrene ("PEG-PS") resin particle at the "R" position. Initially, the boronic acid derivatized particle was loaded with resorufin derivatized galactose 510. Upon exposure of the particle to a solution containing glucose 520, the resorufin derivatized galactose molecules 510 are displaced from the particle receptor sites. Visual inspection of the optical photographs taken before and after exposure to the sugar solution show that the boron substituted resin is capable of sequestering sugar molecules from an aqueous solution. Moreover, the subsequent exposure of the colored particles to a solution of a non-tagged sugar (e.g., glucose) leads to a displacement of the bound colored sugar reporter molecule. Displacement of this molecule leads to a change in the color of the particle. The sugar sensor turns from dark orange to yellow in solutions containing glucose. The particles were also tested in conditions of varying pH. It was noted that the color of the particles changes from dark orange to yellow as the pH is varied from low pH to high pH.

### FURTHER IMPROVEMENTS

Shown in FIG. 17 is an embodiment of a system for detecting analytes in a fluid. In one embodiment, the system includes a light source 512, a sensor array 522, a chamber 550 for supporting the sensor array and a detector 530. The sensor array 522 may include a supporting member which is configured to hold a variety of particles. In one embodiment, light originating from the light source 512 passes through the sensor array 522 and out through the bottom side of the sensor array. Light modulated by the particles may be detected by a proximally spaced detector 530. While depicted as being positioned below the sensor array, it should be understood that the detector may be positioned above the sensor array for reflectance measurements. Evaluation of the optical changes may be completed by visual inspection (e.g., by eye, or with the aid of a microscope) or by use of a microprocessor 540 coupled to the detector.

In this embodiment, the sensor array 522 is positioned within a chamber 550. The chamber 550, may be configured to allow a fluid stream to pass through the chamber such that the fluid stream interacts with the sensor array 522. The chamber may be constructed of glass (e.g., borosilicate glass or quartz) or a plastic material which is transparent to a portion of the light from the light source. If a plastic material is used, the plastic material should also be substantially unreactive toward the fluid. Examples of plastic materials which may be used to form the chamber include, but are not limited to, acrylic resins, polycarbonates, polyester resins, polyethylenes, polyimides, polyvinyl polymers (e.g., polyvinyl chloride, polyvinyl acetate, polyvinyl dichloride, polyvinyl fluoride, etc.), polystyrenes, polypropylenes, polytetrafluoroethylenes, and polyurethanes. An example of such a chamber is a Sykes-Moore chamber, which is commercially available from Bellico Glass, Inc., in New Jersey. Chamber 550, in one embodiment, includes a fluid inlet port 552 and a fluid outlet port 554. The fluid inlet 552 and outlet 554 ports are configured to allow a fluid stream to pass into the interior 556 of the chamber during use. The inlet and outlet ports may be configured to allow facile placement of a conduit for transferring the fluid to the chamber. In one embodiment, the ports may be hollow conduits. The hollow conduits may be configured to have an outer diameter which is substantially equal to the inner diameter of a tube for transferring the fluid to or away from the chamber. For example, if a plastic or rubber tube is used for the transfer of the fluid, the internal diameter of the plastic tube is substantially equal to the outer diameter of the inlet and outlet ports.

In another embodiment, the inlet and outlet ports may be Luer lock style connectors. Preferably, the inlet and outlet ports are female Luer lock connectors. The use of female Luer lock connectors will allow the fluid to be introduced via a syringe. Typically, syringes include a male Luer lock connector at the dispensing end of the syringe. For the introduction of liquid samples, the use of Luer lock connectors may allow samples to be transferred directly from a syringe to the chamber 550. Luer lock connectors may also allow plastic or rubber tubing to be connected to the chamber using Luer lock tubing connectors.

The chamber may be configured to allow the passage of a fluid sample to be substantially confined to the interior 556 of the chamber. By confining the fluid to a small interior volume, the amount of fluid required for an analysis may be minimized. The interior volume may be specifically modified for the desired application. For example, for the analysis of small volumes of fluid samples, the chamber may be designed to have a small interior chamber, thus reducing the amount of fluid needed to fill the chamber. For larger samples, a larger interior chamber may be used. Larger chambers may allow a faster throughput of the fluid during use.

In another embodiment, depicted in FIG. 18, a system for detecting analytes in a fluid includes a light source 512, a sensor array 522, a chamber 550 for supporting the sensor array and a detector 530, all enclosed within a detection system enclosure 560. As described above, the sensor array 522 is preferably formed of a supporting member which is configured to hold a variety of particles. Thus, in a single enclosure, all of the components of an analyte detection system are included.

The formation of an analyte detection system in a single enclosure may allow the formation of a portable detection system. For example, a small controller 570 may be coupled to the analyte detection system. The controller 570 may be configured to interact with the detector and display the results from the analysis. In one embodiment, the controller includes a display device 572 for displaying information to a user. The controller may also include input devices 574 (e.g., buttons) to allow the user to control the operation of the analyte detection

system. For example, the controller may control the operation of the light source 512 and the operation of the detector 530.

The detection system enclosure 560, may be interchangeable with the controller. Coupling members 576 and 578 may be used to remove the detection system enclosure 560 from the controller 570. A second detection system enclosure may be readily coupled to the controller using coupling members 576 and 578. In this manner, a variety of different types of analytes may be detecting using a variety of different detection system enclosures. Each of the detection system enclosures may include different sensor arrays mounted within their chambers. Instead of having to exchange the sensor array for different types of analysis, the entire detection system enclosure may be exchanged. This may prove advantageous, when a variety of detection schemes are used. For example a first detection system enclosure may be configured for white light applications. The first detection system enclosure may include a white light source, a sensor that includes particles that produce a visible light response in the presence of an analyte, and a detector sensitive to white light. A second detection system enclosure may be configured for fluorescent applications, including a fluorescent light source, a sensor array which includes particles which produce a fluorescent response on the presence of an analyte, and a fluorescent detector. The second detection system enclosure may also include other components necessary for producing a proper detection system. For example, the second detection system may also include a filter for preventing short wavelength excitation from producing "false" signals in the optical detection system during fluorescence measurements. A user need only select the proper detection system enclosure for the detection of the desired analyte. Since each detection system enclosure includes many of the required components, a user does not have to make light source selections, sensor array selections or detector arrangement selections to produce a viable detection system.

In another embodiment, the individual components of the system may be interchangeable. The system may include coupling members 573 and 575 that allow the light source and the detector, respectively, to be removed from the chamber 550. This may allow a more modular design of the system. For example, an analysis may be first performed with a white light source to give data corresponding to an absorbance/reflectance analysis. After this analysis is performed the light source may be changed to a ultraviolet light source to allow ultraviolet analysis of the particles. Since the particles have already been treated with the fluid, the analysis may be preformed without further treatment of the particles with a fluid. In this manner a variety of tests may be performed using a single sensor array.

In one embodiment, the supporting member is made of any material capable of supporting the particles, while allowing the passage of the appropriate wavelength of light. The supporting member may also be made of a material substantially impervious to the fluid in which the analyte is present. A variety of materials may be used including plastics (e.g., photoresist materials, acrylic polymers, carbonate polymers, etc.), glass, silicon based materials (e.g., silicon, silicon dioxide, silicon nitride, etc.) and metals. In one embodiment, the supporting member includes a plurality of cavities. The cavities are preferably formed such that at least one particle is substantially contained within the cavity. Alternatively, a plurality of particles may be contained within a single cavity.

In some embodiments, it will be necessary to pass liquids over the sensor array. The dynamic motion of liquids across the sensor array may lead to displacement of the particles from the cavities. In another embodiment, the particles are preferably held within cavities formed in a supporting member by the use of a transmission electron microscope ("TEM") grid. As depicted in FIG. 19, a cavity 580 is formed in a supporting member 582.

After placement of a particle 584 within the cavity, a TEM grid 586 may be placed atop the supporting member 582 and secured into position. TEM grids and adhesives for securing TEM grids to a support are commercially available from Ted Pella, Inc., Redding, CA. The TEM grid 586 may be made from a number of materials including, but not limited to, copper, nickel, gold, silver, aluminum, molybdenum, titanium, nylon, beryllium, carbon, and beryllium-copper. The mesh structure of the TEM grid may allow solution access as well as optical access to the particles that are placed in the cavities. FIG. 20 further depicts a top view of a sensor array with a TEM grid 586 formed upon the upper surface of the supporting member 582. The TEM grid 586 may be placed on the upper surface of the supporting member, trapping particles 584 within the cavities 580. As depicted, the openings 588 in the TEM grid 586 may be sized to hold the particles 584 within the cavities 580, while allowing fluid and optical access to cavities 580.

In another embodiment, a sensor array includes a supporting member configured to support the particles, while allowing the passage of the appropriate wavelength of light to the particle. The supporting member, in one embodiment, includes a plurality of cavities. The cavities may be formed such that at least one particle is substantially contained within the cavity. The supporting member may be configured to substantially inhibit the displacement of the particles from the cavities during use. The supporting member may also be configured to allow the passage of the fluid through cavities, e.g., the fluid may flow from the top surface of the supporting member, past the particle, and out the bottom surface of the supporting member. This may increase the contact time between the particle and the fluid.

Figures 21A-G depict a sequence of processing steps for the formation of a silicon based supporting member which includes a removable top cover and bottom cover. The removable top cover may be configured to allow fluids to pass through the top cover and into the cavity. The removable bottom cover may also be configured to allow the fluid to pass through the bottom cover and out of the cavity. As depicted in FIG. 21A, a series of layers may be deposited upon both sides of a silicon substrate 610. First removable layers 612 may be deposited upon the silicon substrate. The removable layers 612 may be silicon dioxide, silicon nitride, or photoresist material. In one embodiment, a layer of silicon dioxide 612 is deposited upon both surfaces of the silicon substrate 610. Upon these removable layers, covers 614 may be formed. In one embodiment, covers 614 are formed from a material that differs from the material used to form the removable layers 612 and which is substantially transparent to the light source of a detection system. For example, if the removable layers 612 are formed from silicon dioxide, the cover may be formed from silicon nitride. Second removable layers 616 may be formed upon the covers 614. Second removable layers 616 may be formed from a material that differs from the material used to form the covers 614. Second removable layers 616 may be formed from a material similar to the material used to form the first removable layers 612. In one embodiment, first and second removable layers 612 and 616 are formed from silicon dioxide and covers 614 are formed from silicon nitride. The layers are patterned and etched using standard photolithographic techniques. In one embodiment, the remaining portions of the layers are substantially aligned in the position where the cavities are to be formed in the silicon substrate 610.

After the layers have been etched, spacer structures may be formed on the sidewalls of the first removable layers 612, the covers 614, and the second removable layers 616, as depicted in FIG. 21B. The spacer structures may be formed from the same material used to form the second removable layers 616. In one embodiment, depositing a spacer layer of the appropriate material and subjecting the material to an anisotropic etch may form the

spacer structures. An anisotropic etch, such as a plasma etch, employs both physical and chemical removal mechanisms. Ions are typically bombarded at an angle substantially perpendicular to the semiconductor substrate upper surface. This causes substantially horizontal surfaces to be removed faster than substantially vertical surfaces. During this etching procedure the spacer layers are preferably removed such that the only regions of the spacer layers that remain may be those regions near substantially vertical surfaces, e.g., spacer structures 618.

After formation of the spacer structures 618, cover support structures 620, depicted in FIG. 21C, may be formed. The cover support structures may be initially formed by depositing a support structure layer upon the second removable layer 616 and spacer structures 618. The support structure layer is then patterned and etched, using standard photolithography, to form the support structures 620. In one embodiment, the support structures are formed from a material that differs from the removable layers material. In one embodiment, the removable layers may be formed from silicon dioxide while the support structures and covers may be formed from silicon nitride.

Turning to FIG. 21 D, the second removable layers 616 and an upper portion of the spacer structures 618 are preferably removed using a wet etch process. Removal of the second removable layers leaves the top surface of the covers 614 exposed. This allows the covers to be patterned and etched such that openings 622 are formed extending through the covers. These openings 622 may be formed in the covers 614 to allow the passage of fluid through the cover layers. In one embodiment, the openings 622 are formed to allow fluid to pass through, while inhibiting displacement of the particles from the subsequently formed cavities.

After the openings 622 have been formed, the remainder of the first removable layers 612 and the remainder of the spacer structures 618 may be removed using a wet etch. The removal of the removable layers and the spacer structures creates "floating" covers 614, as depicted in FIG. 21E. The covers 614 may be held in proximity to the silicon substrate 610 by the support structures 620. The covers 614 may now be removed by sliding the covers away from the support structures 620. In this manner removable covers 614 may be formed.

After the covers 614 are removed, cavities 640 may be formed in the silicon substrate 610, as depicted in FIG. 21F. The cavities 640 may be formed by, initially patterning and etching a photoresist material 641 to form a masking layer. After the photoresist material 641 is patterned, the cavities 640 may be etched into the silicon substrate 610 using a hydroxide etch, as described previously.

After the cavities 640 are formed, the photoresist material may be removed and particles 642 may be placed within the cavities, as depicted in FIG. 21G. The particles 642, may be inhibited from being displaced from the cavity 640 by placing covers 614 back onto the upper and lower faces of the silicon substrate 610.

In another embodiment, a sensor array may be formed using a supporting member, a removable cover, and a secured bottom layer. FIGS. 22 A-G depict a series of processing steps for the formation of a silicon based supporting member which includes a removable top cover and a secured bottom layer. The removable top cover is preferably configured to allow fluids to pass through the top cover and into the cavity. As depicted in FIG. 22A, a series of layers may be deposited upon both sides of a silicon substrate 610. A first removable layer 612 may be deposited upon the upper face 611 of the silicon substrate 610. The removable layer 612 may be silicon dioxide, silicon nitride, or photoresist material. In one embodiment, a layer of silicon dioxide 612 is deposited upon the silicon substrate 610. A cover 614 may be formed upon the removable layer 612 of the silicon substrate 610. In one embodiment, the cover 614 is formed from a material that differs from the material used to form the removable layer 612 and is substantially transparent to the light source of a detection system. For example, if the removable

layer 612 is formed from silicon dioxide, the cover layer 614 may be formed from silicon nitride. In one embodiment, a bottom layer 615 is formed on the bottom surface 613 of the silicon substrate 610. In one embodiment, the bottom layer 615 is formed from a material that is substantially transparent to the light source of a detection system. A second removable layer 616 may be formed upon the cover 614. Second removable layer 616  
5 may be formed from a material that differs from the material used to form the cover layer 614. Second removable layer 616 may be formed from a material similar to the material used to form the first removable layer 612. In one embodiment, first and second removable layers 612 and 616 are formed from silicon dioxide and cover 614 is formed from silicon nitride. The layers formed on the upper surface 611 of the silicon substrate may be patterned and etched using standard photolithographic techniques. In one embodiment, the remaining portions of the layers  
10 formed on the upper surface are substantially aligned in the position where the cavities are to be formed in the silicon substrate 610.

After the layers have been etched, spacer structures may be formed on the side walls of the first removable layer 612, the cover 614, and the second removable layer 616, as depicted in FIG. 22B. The spacer structures may be formed from the same material used to form the second removable layer 616. In one embodiment, the spacer  
15 structures may be formed by depositing a spacer layer of the appropriate material and subjecting the spacer layer to an anisotropic etch. During this etching procedure the spacer layer is preferably removed such that the only regions of the spacer layer which remain may be those regions near substantially vertical surfaces, e.g., spacer structures 618.

After formation of the spacer structures 618, cover support structures 620, depicted in FIG. 22C, may be  
20 formed upon the removable layer 616 and the spacer structures 618. The cover support structures 620 may be formed by depositing a support structure layer upon the second removable layer 616 and spacer structures 618. The support structure layer is then patterned and etched, using standard photolithography, to form the support structures 620. In one embodiment, the support structures are formed from a material that differs from the removable layer materials. In one embodiment, the removable layers may be formed from silicon dioxide while the support  
25 structures and cover may be formed from silicon nitride.

Turning to FIG. 22 D, the second removable layer 616 and an upper portion of the spacer structures 618 may be removed using a wet etch process. Removal of the second removable layer leaves the top surface of the cover 614 exposed. This allows the cover 614 to be patterned and etched such that openings 622 are formed  
30 extending through the cover 614. These openings 622 may be formed in the cover 614 to allow the passage of fluid through the cover. In one embodiment, the openings 622 are formed to allow fluid to pass through, while inhibiting displacement of the particle from a cavity. The bottom layer 615 may also be similarly patterned and etched such that openings 623 may be formed extending thorough the bottom layer 615.

After the openings 622 and 623 are formed, the first removable layer 612 and the remainder of the spacer structures 618 may be removed using a wet etch. The removal of the removable layers and the spacer structures  
35 creates a "floating" cover 614, as depicted in FIG. 22E. The cover 614 may be held in proximity to the silicon substrate 610 by the support structures 620. The cover 614 may now be removed by sliding the cover 614 away from the support structures 620. In this manner a removable cover 614 may be formed.

After the cover 614 is removed, cavities 640 may be formed in the silicon substrate 610, as depicted in FIG. 22F. The cavities 640 may be formed by, initially patterning and etching a photoresist material 641 to form a



masking layer. After the photoresist material 614 is patterned, the cavities 640 may be etched into the silicon substrate 610 using a hydroxide etch, as described previously.

After the cavities 640 are formed, the photoresist material may be removed and particles 642 may be placed within the cavities, as depicted in FIG. 22G. The particles 642, may be inhibited from being displaced from the cavity 640 by placing cover 614 back onto the upper face 611 of the silicon substrate 610. The bottom layer 615 may also aid in inhibiting the particle 642 from being displaced from the cavity 640. Openings 622 in cover 614 and openings 623 in bottom layer 615 may allow fluid to pass through the cavity during use.

In another embodiment, a sensor array may be formed using a supporting member and a removable cover. FIGS. 23A-G depict a series of processing steps for the formation of a silicon based supporting member which includes a removable cover. The removable cover is preferably configured to allow fluids to pass through the cover and into the cavity. As depicted in FIG. 23A, a series of layers may be deposited upon the upper surface 611 of a silicon substrate 610. A first removable layer 612 may be deposited upon the upper face 611 of the silicon substrate 610. The removable layer 612 may be silicon dioxide, silicon nitride, or photoresist material. In one embodiment, a layer of silicon dioxide 612 is deposited upon the silicon substrate 610. A cover 614 may be formed upon the removable layer 612. In one embodiment, the cover is formed from a material which differs from the material used to form the removable layer 612 and which is substantially transparent to the light source of a detection system. For example, if the removable layer 612 is formed from silicon dioxide, the cover 614 may be formed from silicon nitride. A second removable layer 616 may be formed upon the cover 614. Second removable layer 616 may be formed from a material that differs from the material used to form the cover 614. Second removable layer 616 may be formed from a material similar to the material used to form the first removable layer 612. In one embodiment, first and second removable layers 612 and 616 are formed from silicon dioxide and cover 614 is formed from silicon nitride. The layers formed on the upper surface 611 of the silicon substrate may be patterned and etched using standard photolithographic techniques. In one embodiment, the remaining portions of the layers formed on the upper surface are substantially aligned in the position where the cavities are to be formed in the silicon substrate 610.

After the layers have been etched, spacer structures 618 may be formed on the side walls of the first removable layer 612, the cover layer 614, and the second removable layer 616, as depicted in FIG. 23B. The spacer structures 618 may be formed from the same material used to form the second removable layer 616. In one embodiment, the spacers may be formed by depositing a spacer layer of the appropriate material upon the second removable layer and subjecting the material to an anisotropic etch. During this etching procedure the spacer layer is preferably removed such that the only regions of the spacer layer which remain may be those regions near substantially vertical surfaces, e.g., spacer structures 618.

After formation of the spacer structures 618, cover support structures 620, depicted in FIG. 23C, may be formed upon the removable layer 616 and the spacer structures 618. The cover support structure may be formed by initially depositing a support structure layer upon the second removable layer 616 and spacer structures 618. The support structure layer is then patterned and etched, using standard photolithography, to form the support structures 620. In one embodiment, the support structures 620 are formed from a material that differs from the removable layer materials. In one embodiment, the removable layers may be formed from silicon dioxide while the support structure and cover layer may be formed from silicon nitride.

Turning to FIG. 23D, the second removable layer 616 and an upper portion of the spacer structures 618 may be removed using a wet etch process. Removal of the second removable layer leaves the top surface of the cover 614 exposed. This allows the cover 614 to be patterned and etched such that openings 622 are formed extending through the cover 614. These openings 622 may be formed in the cover 614 to allow the passage of fluid through the cover 614.

After the openings 622 are formed, the remainder of the first removable layer 612 and the remainder of the spacer structures 618 may be removed using a wet etch. The removal of the removable layers and the spacer structures creates a "floating" cover 614, as depicted in FIG. 23E. The cover 614 is preferably held in proximity to the silicon substrate 610 by the support structures 620. The cover 614 may now be removed by sliding the cover 614 away from the support structures 620. In this manner a removable cover 614 may be formed.

After the cover 614 is removed, cavities 640 may be formed in the silicon substrate 610, as depicted in FIG. 23F. The cavities 640 may be formed by initially depositing and patterning a photoresist material 641 upon the silicon support 610. After the photoresist material 641 is patterned, the cavities 640 may be etched into the silicon substrate 610 using a hydroxide etch, as described previously. The etching of the cavities may be accomplished such that a bottom width of the cavity 643 is less than a width of a particle 642. In one embodiment, the width of the bottom of the cavity may be controlled by varying the etch time. Typically, longer etching times result in a larger opening at the bottom of the cavity. By forming a cavity in this manner, a particle placed in the cavity may be too large to pass through the bottom of the cavity. Thus, a supporting member that does not include a bottom layer may be formed. An advantage of this process is that the processing steps may be reduced making production simpler.

After the cavities 640 are formed, the photoresist material may be removed and particles 642 may be placed within the cavities, as depicted in FIG. 23G. The particles 642, may be inhibited from being displaced from the cavity 640 by placing cover 614 back onto the upper face 611 of the silicon substrate 610. The narrow bottom portion of the cavity may also aid in inhibiting the particle 642 from being displaced from the cavity 640.

Figures 24A-d depict a sequence of processing steps for the formation of a silicon based supporting member which includes a top partial cover and a bottom partial cover. The top partial cover and bottom partial covers are, in one embodiment, configured to allow fluids to pass into the cavity and out through the bottom of the cavity. As depicted in FIG. 24A, a bottom layer 712 may be deposited onto the bottom surface of a silicon substrate 710. The bottom layer 712 may be silicon dioxide, silicon nitride, or photoresist material. In one embodiment, a layer of silicon nitride 712 is deposited upon the silicon substrate 710. In one embodiment, openings 714 are formed through the bottom layer as depicted in FIG. 24A. Openings 714, in one embodiment, are substantially aligned with the position of the cavities to be subsequently formed. The openings 714 may have a width that is substantially less than a width of a particle. Thus a particle will be inhibited from passing through the openings 714.

Cavities 716 may be formed in the silicon substrate 710, as depicted in FIG. 24B. The cavities 716 may be formed by initially depositing and patterning a photoresist layer upon the silicon substrate 710. After the photoresist material is patterned, cavities 716 may be etched into the silicon substrate 710 using a number of etching techniques, including wet and plasma etches. The width of the cavities 716 is preferably greater than the width of a particle, thus allowing a particle to be placed within each of the cavities. The cavities 716, in one

embodiment, are preferably formed such that the cavities are substantially aligned over the openings 714 formed in the bottom layer.

After the cavities have been formed, particles 718 may be inserted into the cavities 716, as depicted in FIG. 24C. The etched bottom layer 712 may serve as a support for the particles 718. Thus the particles 718 may  
5 be inhibited from being displaced from the cavities by the bottom layer 712. The openings 714 in the bottom layer 712 may allow fluid to pass through the bottom layer during use.

After the particles are placed in the cavities, a top layer 720 may be placed upon the upper surface 717 of the silicon substrate. In one embodiment, the top layer 720 is formed from a material is substantially transparent to the light source of a detection system. The top layer may be formed from silicon nitride, silicon dioxide or  
10 photoresist material. In one embodiment, a sheet of photoresist material is used. After the top layer 620 is formed, openings 719 may be formed in the top layer to allow the passage of the fluid into the cavities. If the top layer 720 is composed of photoresist material, after depositing the photoresist material across the upper surface of the silicon substrate, the openings may be initially formed by exposing the photoresist material to the appropriate wavelength and pattern of light. If the top layer is composed of silicon dioxide or silicon nitride the top layer 720 may be  
15 developed by forming a photoresist layer upon the top layer, developing the photoresist, and using the photoresist to etch the underlying top layer.

Similar sensor arrays may be produced using materials other than silicon for the supporting member. For example, as depicted in FIG 25 A-D, the supporting member may be composed of photoresist material. In one embodiment, sheets of photoresist film may be used to form the supporting member. Photoresist film sheets are  
20 commercially available from E. I. du Pont de Nemours and Company, Wilmington, DE under the commercial name RISTON. The sheets come in a variety of sizes, the most common having a thickness ranging from about 1 mil. (25  $\mu\text{m}$ ) to about 2 mil. (50  $\mu\text{m}$ ).

In an embodiment, a first photoresist layer 722 is developed and etched such that openings 724 are formed. The openings may be formed proximate the location of the subsequently formed cavities. Preferably, the  
25 openings have a width that is substantially smaller than a width of the particle. The openings may inhibit displacement of the particle from a cavity. After the first photoresist layer 720 is patterned and etched, a main layer 726 is formed upon the bottom layer. The main layer 720 is preferably formed from a photoresist film that has a thickness substantially greater than a typical width of a particle. Thus, if the particles have a width of about 30  $\mu\text{m}$ , a main layer may be composed of a 50  $\mu\text{m}$  photoresist material. Alternatively, the photoresist layer may be  
30 composed of a multitude of photoresist layers placed upon each other until the desired thickness is achieved, as will be depicted in later embodiments.

The main photoresist layer may be patterned and etched to form the cavities 728, as depicted in FIG. 25B. The cavities, in one embodiment, are substantially aligned above the previously formed openings 724. Cavities 728, in one embodiment, have a width which is greater than a width of a particle.

35 For many types of analysis, the photoresist material is substantially transparent to the light source used. Thus, as opposed to a silicon supporting member, the photoresist material used for the main supporting layer may be substantially transparent to the light used by the light source. In some circumstances, the transparent nature of the supporting member may allow light from the cavity to migrate, through the supporting member, into a second cavity. This leakage of light from one cavity to the next may lead to detection problems. For example, if a first

particle in a first cavity produces a fluorescent signal in response to an analyte, this signal may be transmitted through the supporting member and detected in a proximate cavity. This may lead to inaccurate readings for the proximately spaced cavities, especially if a particularly strong signal is produced by the interaction of the particle with an analyte.

5 To reduce the occurrence of this "cross-talk", a substantially reflective layer 730 may be formed along the inner surface of the cavity. In one embodiment, the reflective layer 730 is composed of a metal layer which is formed on the upper surface of the main layer and the inner surface of the cavity. The metal layer may be deposited using chemical vapor deposition or other known techniques for depositing thin metal layers. The presence of a reflective layer may inhibit "cross-talk" between the cavities.

10 After the cavities 728 have been formed, particles 718 may be inserted into the cavities 728, as depicted in FIG. 25C. The first photoresist layer 722 may serve as a support for the particles 718. The particles may be inhibited from being displaced from the cavities by the first photoresist layer 722. The openings 724 in the first photoresist layer 722 may allow fluid to pass through the bottom layer during use.

15 After the particles 728 are placed in the cavities 728, a top photoresist layer 732 may be placed upon the upper surface of the silicon substrate. After the cover layer is formed, openings 734 may be formed in the cover layer to allow the passage of the fluid into the cavities.

In another embodiment, the supporting member may be formed from a plastic substrate, as depicted in FIG. 26A-D. In one embodiment, the plastic substrate is composed of a material which is substantially resistant to the fluid which includes the analyte. Examples of plastic materials which may be used to form the plastic substrate include, but are not limited to, acrylic resins, polycarbonates, polyester resins, polyethylenes, polyimides, polyvinyl polymers (e.g., polyvinyl chloride, polyvinyl acetate, polyvinyl dichloride, polyvinyl fluoride, etc.), polystyrenes, polypropylenes, polytetrafluoroethylenes, and polyurethanes. The plastic substrate may be substantially transparent or substantially opaque to the light produced by the light source. After obtaining a suitable plastic material 740, a series of cavities 742 may be formed in the plastic material. The cavities 740 may be formed by drilling (either  
20 mechanically or with a laser), transfer molding (e.g., forming the cavities when the plastic material is formed using appropriately shaped molds), or using a punching apparatus to punch cavities into the plastic material. In one embodiment, the cavities 740 are formed such that a lower portion 743 of the cavities is substantially narrower than an upper portion 744 of the cavities. The lower portion 743 of the cavities may have a width substantially less than a width of a particle. The lower portion 743 of the cavities 740 may inhibit the displacement of a particle from the cavity 740. While depicted as rectangular, with a narrower rectangular opening at the bottom, it should be  
25 understood that the cavity may be formed in a number of shapes including but not limited to pyramidal, triangular, trapezoidal, and oval shapes. An example of a pyramidal cavity which is tapered such that the particle is inhibited from being displaced from the cavity is depicted in FIG. 25D.

30 After the cavities 742 are formed, particles 718 may be inserted into the cavities 742, as depicted in FIG. 26B. The lower portion 743 of the cavities may serve as a support for the particles 718. The particles 718 may be inhibited from being displaced from the cavities 742 by the lower portion 743 of the cavity. After the particles are placed in the cavities 740, a cover 744 may be placed upon the upper surface 745 of the plastic substrate 740, as depicted in FIG. 26C. In one embodiment, the cover is formed from a film of photoresist material. After the cover  
35

744 is placed on the plastic substrate 740, openings 739 may be formed in the cover layer to allow the passage of the fluid into the cavities.

In some circumstances a substantially transparent plastic material may be used. As described above, the use of a transparent supporting member may lead to "cross-talk" between the cavities. To reduce the occurrence of this "cross-talk", a substantially reflective layer 748 may be formed on the inner surface 746 of the cavity, as depicted in FIG. 26E. In one embodiment, the reflective layer 748 is composed of a metal layer which is formed on the inner surface of the cavities 742. The metal layer may be deposited using chemical vapor deposition or other techniques for depositing thin metal layers. The presence of a reflective layer may inhibit cross-talk between the cavities.

In another embodiment, a silicon based supporting member for a sensing particle may be formed without a bottom layer. In this embodiment, the cavity may be tapered to inhibit the passage of the particle from the cavity, through the bottom of the supporting member. FIG. 27A-D, depicts the formation of a supporting member from a silicon substrate. In this embodiment, a photoresist layer 750 is formed upon an upper surface of a silicon substrate 752, as depicted in FIG. 27A. The photoresist layer 750 may be patterned and developed such that the regions of the silicon substrate in which the cavities will be formed are exposed.

Cavities 754 may now be formed, as depicted in FIG. 27B, by subjecting the silicon substrate to an anisotropic etch. In one embodiment, a potassium hydroxide etch is used to produce tapered cavities. The etching may be controlled such that the width of the bottom of the cavities 750 is less than a width of the particle. After the cavities have been etched, a particle 756 may be inserted into the cavities 754 as depicted in FIG. 27C. The particle 756 may be inhibited from passing out of the cavities 754 by the narrower bottom portion of the cavities. After the particle is positioned within the cavities 754, a cover 758 may be formed upon the silicon substrate 752, as depicted in FIG. 27D. The cover may be formed of any material substantially transparent to the light produced by the light source used for analysis. Openings 759 may be formed in the cover 758 to allow the fluid to pass into the cavity from the top face of the supporting member 752. The openings 759 in the cover and the opening at the bottom of the cavities 754 together may allow fluid to pass through the cavity during use.

In another embodiment, a supporting member for a sensing particle may be formed from a plurality of layers of a photoresist material. In this embodiment, the cavity may be tapered to inhibit the passage of the particle from the cavity, through the bottom of the supporting member. FIGS. 28A-E depict the formation of a supporting member from a plurality of photoresist layers. In an embodiment, a first photoresist layer 760 is developed and etched to form a series of openings 762 which are positioned at the bottom of subsequently formed cavities, as depicted in FIG. 28A. As depicted in FIG. 28B, a second layer of photoresist material 764 may be formed upon the first photoresist layer 760. The second photoresist layer may be developed and etched to form openings substantially aligned with the openings of the first photoresist layer 760. The openings formed in the second photoresist layer 764, in one embodiment, are substantially larger than the layers formed in the first photoresist layer 760. In this manner, a tapered cavity may be formed while using multiple photoresist layers.

As depicted in FIG. 28C, additional layers of photoresist material 766 and 768 may be formed upon the second photoresist layer 764. The openings of the additional photoresist layers 766 and 768 may be progressively larger as each layer is added to the stack. In this manner, a tapered cavity may be formed. Additional layers of photoresist material may be added until the desired thickness of the supporting member is obtained. The thickness

of the supporting member, in one embodiment, is greater than a width of a particle. For example, if a layer of photoresist material has a thickness of about 25  $\mu\text{m}$  and a particle has a width of about 100  $\mu\text{m}$ , a supporting member may be formed from four or more layers of photoresist material. While depicted as pyramidal, the cavity may be formed in a number of different shapes, including but not limited to, rectangular, circular, oval, triangular, and trapezoidal. Any of these shapes may be obtained by appropriate patterning and etching of the photoresist layers as they are formed.

In some instances, the photoresist material may be substantially transparent to the light produced by the light source. As described above, the use of a transparent supporting member may lead to "cross-talk" between the cavities. To reduce the occurrence of this "cross-talk", a substantially reflective layer 770 may be formed along the inner surface of the cavities 762, as depicted in FIG. 28D. In one embodiment, the reflective layer is composed of a metal layer which is formed on the inner surface of the cavities 762. The metal layer may be deposited using chemical vapor deposition or other techniques for depositing thin metal layers. The presence of a reflective layer may inhibit "cross-talk" between the cavities.

After the cavities 762 are formed, particles 772 may be inserted into the cavities 762, as depicted in FIG. 28D. The narrow portions of the cavities 762 may serve as a support for the particles 772. The particles 772 may be inhibited from being displaced from the cavities 762 by the lower portion of the cavities. After the particles 772 are placed in the cavities 762, a cover 774 may be placed upon the upper surface of the top layer 776 of the supporting member, as depicted in FIG. 28E. In one embodiment, the cover 774 is also formed from a film of photoresist material. After the cover layer is formed, openings 778 may be formed in the cover 774 to allow the passage of the fluid into the cavities.

In another embodiment, a supporting member for a sensing particle may be formed from photoresist material which includes a particle support layer. FIGS. 29A-E depict the formation of a supporting member from a series of photoresist layers. In an embodiment, a first photoresist layer 780 is developed and etched to form a series of openings 782 which may become part of subsequently formed cavities. In another embodiment, a cavity having the appropriate depth may be formed by forming multiple layers of a photoresist material, as described previously. As depicted in FIG. 29B, a second photoresist layer 784 may be formed upon the first photoresist layer 780. The second photoresist layer 784 may be patterned to form openings substantially aligned with the openings of the first photoresist layer 782. The openings formed in the second photoresist layer 784 may be substantially equal in size to the previously formed openings. Alternatively, the openings may be variable in size to form different shaped cavities.

For reasons described above, a substantially reflective layer 786 may be formed along the inner surface of the cavities 782 and the upper surface of the second photoresist layer 784, as depicted in FIG. 29C. In one embodiment, the reflective layer is composed of a metal layer. The metal layer may be deposited using chemical vapor deposition or other techniques for depositing thin metal layers. The presence of a reflective layer may inhibit "cross-talk" between the cavities.

After the metal layer is deposited, a particle support layer 788 may be formed on the bottom surface of the first photoresist layer 780, as depicted in FIG. 29D. The particle support layer 788 may be formed from photoresist material, silicon dioxide, silicon nitride, glass or a substantially transparent plastic material. The particle support

layer 788 may serve as a support for the particles placed in the cavities 782. The particle support layer, in one embodiment, is formed from a material that is substantially transparent to the light produced by the light source.

After the particle supporting layer 788 is formed, particles 785 may be inserted into the cavities 782, as depicted in FIG. 29E. The particle support layer 788 may serve as a support for the particles. Thus the particles 785 may be inhibited from being displaced from the cavities by the particle support layer 788. After the particles 785 are placed in the cavities 782, a cover 787 may be placed upon the upper surface of the second photoresist layer 784, as depicted in FIG. 29E. In one embodiment, the cover is also formed from a film of photoresist material. After the cover is formed, openings 789 may be formed in the cover 787 to allow the passage of the fluid into the cavities. In this embodiment, the fluid is inhibited from flowing through the supporting member. Instead, the fluid may flow into and out of the cavities via the openings 789 formed in the cover 787.

A similar supporting member may be formed from a plastic material, as depicted in FIGS. 30A-D. The plastic material may be substantially resistant to the fluid which includes the analyte. The plastic material may be substantially transparent or substantially opaque to the light produced by the light source. After obtaining a suitable plastic substrate 790, a series of cavities 792 may be formed in the plastic substrate 790. The cavities may be formed by drilling (either mechanically or with a laser), transfer molding (e.g., forming the cavities when the plastic substrate is formed using appropriately shaped molds), or using a punching machine to form the cavities. In one embodiment, the cavities extend through a portion of the plastic substrate, terminating proximate the bottom of the plastic substrate, without passing through the plastic substrate. After the cavities 792 are formed, particles 795 may be inserted into the cavities 792, as depicted in FIG. 30B. The bottom of the cavity may serve as a support for the particles 795. After the particles are placed in the cavities, a cover 794 may be placed upon the upper surface of the plastic substrate 790, as depicted in FIG. 30C. In one embodiment, the cover may be formed from a film of photoresist material. After the cover 794 is formed, openings 796 may be formed in the cover to allow the passage of the fluid into the cavities. While depicted as rectangular, it should be understood that the cavities may be formed in a variety of different shapes, including triangular, pyramidal, pentagonal, polygonal, oval, or circular. It should also be understood that cavities having a variety of different shapes may be formed into the same plastic substrate, as depicted in FIG. 30D.

In one embodiment, a series of channels may be formed in the supporting member interconnecting some of the cavities, as depicted in FIG. 3. Pumps and valves may also be incorporated into the supporting member to aid passage of the fluid through the cavities. A schematic figure of a diaphragm pump 800 is depicted in FIG. 31. Diaphragm pumps, in general, include a cavity 810, a flexible diaphragm 812, an inlet valve 814, and an outlet valve 816. The flexible diaphragm 812, during use, is deflected as shown by arrows 818 to create a pumping force. As the diaphragm is deflected toward the cavity 810 it may cause the inlet valve 814 to close, the outlet valve 816 to open and any liquid which is in the cavity 810 will be forced toward the outlet 816. As the diaphragm moves away from the cavity 810, the outlet valve 816 may be pulled to a closed position, and the inlet valve 814 may be opened, allowing additional fluid to enter the cavity 810. In this manner a pump may be used to pump fluid through the cavities. It should be understood that the pump depicted in FIG. 31 is a generalized version of a diaphragm based pump. Actual diaphragm pumps may have different shapes or may have inlet and outlet valves which are separate from the pumping device.

In one embodiment, the diaphragm 810 may be made from a piezoelectric material. This material will contract or expand when an appropriate voltage is applied to the diaphragm. Pumps using a piezoelectric diaphragms are described in U.S. Patent Nos. 4,344,743, 4,938,742, 5,611,676, 5,705,018, and 5,759,015, all of which are incorporated by reference. In other embodiments, the diaphragm may be activated using a pneumatic system. In these systems, an air system may be coupled to the diaphragm such that changes in air density about the diaphragm, induced by the pneumatic system, may cause the diaphragm to move toward and away from the cavity. A pneumatically controlled pump is described in United States Patent No. 5,499,909 which is incorporated by reference. The diaphragm may also be controlled using a heat activated material. The diaphragm may be formed from a temperature sensitive material. In one embodiment, the diaphragm may be formed from a material which is configured to expand and contract in response to temperature changes. A pump system which relies on temperature activated diaphragm is described in United States Patent No. 5,288,214 which is incorporated by reference.

In another embodiment, an electrode pump system may be used. FIG. 32 depicts a typical electrode based system. A series of electrodes 820 may be arranged along a channel 822 which may lead to a cavity 824 which includes a particle 826. By varying the voltage in the electrodes 820 a current flow may be induced in the fluid within the channel 822. Examples of electrode based systems include, but are not limited to, electroosmosis systems, electrohydrodynamic systems, and combinations of electroosmosis and electrohydrodynamic systems.

Electrohydrodynamic pumping of fluids is known and may be applied to small capillary channels. In an electrohydrodynamic system electrodes are typically placed in contact with the fluid when a voltage is applied. The applied voltage may cause a transfer in charge either by transfer or removal of an electron to or from the fluid. This electron transfer typically induces liquid flow in the direction from the charging electrode to the oppositely charged electrode. Electrohydrodynamic pumps may be used for pumping fluids such as organic solvents.

Electroosmosis, is a process which involves applying a voltage to a fluid in a small space, such as a capillary channel, to cause the fluid to flow. The surfaces of many solids, including quartz, glass and the like, become variously charged, negatively or positively, in the presence of ionic materials, such as for example salts, acids or bases. The charged surfaces will attract oppositely charged (positive or negative) counterions in aqueous solutions. The application of a voltage to such a solution results in a migration of the counterions to the oppositely charged electrode, and moves the bulk of the fluid as well. The volume flow rate is proportional to the current, and the volume flow generated in the fluid is also proportional to the applied voltage. An electroosmosis pump system is described in United States Patent No. 4,908,112 which is incorporated by reference.

In another embodiment, a combination of electroosmosis pumps and electrohydrodynamic pumps may be used. Wire electrodes may be inserted into the walls of a channel at preselected intervals to form alternating electroosmosis and electrohydrodynamic devices. Because electroosmosis and electrohydrodynamic pumps are both present, a plurality of different solutions, both polar and non-polar, may be pump along a single channel. Alternatively, a plurality of different solutions may be passed along a plurality of different channels connected to a cavity. A system which includes a combination of electroosmosis pumps and electrohydrodynamic pumps is described in United States Patent No. 5,632,876 which is incorporated by reference.

In an embodiment, a pump may be incorporated into a sensor array system, as depicted in FIG. 32. A sensor array 830 includes at least one cavity 832 in which a particle 834 may be placed. The cavity 832 may be configured to allow fluid to pass through the cavity during use. A pump 836 may be incorporated onto a portion of



the supporting member 838. A channel 831 may be formed in the supporting member 838 coupling the pump 836 to the cavity 832. The channel 831 may be configured to allow the fluid to pass from the pump 836 to the cavity 832. The pump 836 may be positioned away from the cavity 832 to allow light to be directed through the cavity during use. The supporting member 838 and the pump 836 may be formed from a silicon substrate, a plastic material, or photoresist material. The pump 836 may be configured to pump fluid to the cavity via the channel, as depicted by the arrows in FIG. 32. When the fluid reaches the cavity 832, the fluid may flow past the particle 834 and out through the bottom of the cavity. An advantage of using pumps is that better flow through the channels may be achieved. Typically, the channels and cavities may have a small volume. The small volume of the cavity and channel tends to inhibit flow of the fluid through the cavity. By incorporating a pump, the flow of fluid to the cavity and through the cavity may be increased, allowing more rapid testing of the fluid sample. While a diaphragm based pump system is depicted in FIG. 33, it should be understood that electrode based pumping systems may also be incorporated into the sensor array to produce fluid flows.

In another embodiment, a pump may be coupled to a supporting member for analyzing analytes in a fluid stream, as depicted in FIG. 34. A channel 842 may couple a pump 846 to multiple cavities 844 formed in a supporting member 840. The cavities 842 may include sensing particles 848. The pump may be configured to create a flow of the fluid through the channel 842 to the cavities 848. In one embodiment, the cavities may inhibit the flow of the fluid through the cavities 844. The fluid may flow into the cavities 844 and past the particle 848 to create a flow of fluid through the sensor array system. In this manner a single pump may be used to pass the fluid to multiple cavities. While a diaphragm pump system is depicted in FIG. 33, it should be understood that electrode pumping systems may also be incorporated into the supporting member to create similar fluid flows..

In another embodiment, multiple pumps may be coupled to a supporting member of a sensor array system. In one embodiment, the pumps may be coupled in series with each other to pump fluid to each of the cavities. As depicted in FIG. 35, a first pump 852 and a second pump 854 may be coupled to a supporting member 850. The first pump 852 may be coupled to a first cavity 856. The first pump may be configured to transfer fluid to the first cavity 856 during use. The cavity 856 may be configured to allow the fluid to pass through the cavity to a first cavity outlet channel 858. A second pump 854 may also be coupled to the supporting member 850. The second pump 854 may be coupled to a second cavity 860 and the first cavity outlet channel 858. The second pump 854 may be configured to transfer fluid from the first cavity outlet channel 858 to the second cavity 860. The pumps may be synchronized such that a steady flow of fluid through the cavities is obtained. Additional pumps may be coupled to the second cavity outlet channel 862 such that the fluid may be pumped to additional cavities. In one embodiment, each of the cavities in the supporting member is coupled to a pump configured to pump the fluid stream to the cavity.

In another embodiment, multiple electrode based pumps may be incorporated into the sensor array system. The pumps may be formed along the channels which couple the cavities. . As depicted in FIG. 36, a plurality of cavities 870 may be formed in a supporting member 872 of a sensor array. Channels 874 may also be formed in the supporting member 872 interconnecting the cavities 870 with each other. An inlet channel 876 and an outlet channel 877, which allow the fluid to pass into and out of the sensor array, respectively, may also be formed. A series of electrodes 878 may be positioned over the channels 874, 876, and 877. The electrodes may be used to form an electroosmosis pumping system or an electrohydrodynamic pumping system. The electrodes may

be coupled to a controller 880 which may apply the appropriate voltage to the appropriate electrodes to produce a flow of the fluid through the channels. The pumps may be synchronized such that a steady flow of fluid through the cavities is obtained. The electrodes may be positioned between the cavities such that the electrodes do not significantly interfere with the application of light to the cavities.

5 In some instances it may be necessary to add a reagent to a particle before, during or after an analysis process. Reagents may include receptor molecules or indicator molecules. Typically, such reagents may be added by passing a fluid stream which includes the reagent over the sensor array. In an embodiment, the reagent may be incorporated into the sensor array system which includes two particles. In this embodiment, a sensor array system 900 may include two particles 910 and 920 for each sensing position of the sensor array, as depicted in FIG. 37.

10 The first particle 910 may be positioned in a first cavity 912. The second particle 920 may be positioned in a second cavity 922. In one embodiment, the second cavity is coupled to the first cavity via a channel 930. The second particle includes a reagent which is at least partially removable from the second particle 920. The reagent may also be configured to modify the first particle 910, when the reagent is contacted with the first particle, such that the first particle will produce a signal when the first particle interacts with an analyte during use. The reagent  
15 may be added to the first cavity before, during or after a fluid analysis. The reagent is preferably coupled to the second particle 920. The a portion of the reagent coupled to the second particle may be decoupled from the particle by passing a decoupling solution past the second particle. The decoupling solution may include a decoupling agent which will cause at least a portion of the reagent to be at released by the particle. A reservoir 940 may be formed on the sensor array to hold the decoupling solution.

20 A first pump 950 and a second pump 960 may also be coupled to the supporting member 915. The first pump 950 may be configured to pump fluid from a fluid inlet 952 to the first cavity 912 via channel 930. The fluid inlet 952 is the location where the fluid, which includes the analyte, is introduced into the sensor array system. A second pump 950 may be coupled to the reservoir 940 and the second cavity 922. The second pump 960 may be used to transfer the decoupling solution from the reservoir to the second cavity 922. The decoupling solution may  
25 pass through the second cavity 922 and into first cavity 912. Thus, as the reagent is removed the second particle it may be transferred to the first cavity 912, where the reagent may interact with the first particle 910. The reservoir may be refilled by removing the reservoir outlet 942, and adding additional fluid to the reservoir 940. While diaphragm based pump systems are depicted in FIG. 37, it should be understood that electrode based pumping systems may also be incorporated into the sensor array to produce fluid flows.

30 The use of such a system is described by way of example. In some instances it may be desirable to add a reagent to the first particle prior to passing the fluid which includes the analyte to the first particle. The reagent may be coupled to the second particle and placed in the sensor array prior to use, typically during construction of the array. A decoupling solution may be added to the reservoir before use. A controller 970 may also be coupled to the system to allow automatic operation of the pumps. The controller 970 may be configured to initiate the  
35 analysis sequence by activating the second pump 960, causing the decoupling solution to flow from the reservoir 940 to the second cavity 922. As the fluid passes through the second cavity 922, the decoupling solution may cause at least some of the reagent molecules to be released from the second particle 920. The decoupling solution may be passed out of the second cavity 922 and into the first cavity 912. As the solution passes through the first cavity, some of the reagent molecules may be captured by the first particle 910. After a sufficient number of molecules

have been captured by the first particle 910, flow of fluid through the second cavity 922 may be stopped. During this initialization of the system, the flow of fluid through the first pump may be inhibited.

After the system is initialized, the second pump may be stopped and the fluid may be introduced to the first cavity. The first pump may be used to transfer the fluid to the first cavity. The second pump may remain off, thus inhibiting flow of fluid from the reservoir to the first cavity. It should be understood that the reagent solution may be added to the first cavity while the fluid is added to the first cavity. In this embodiment, both the first and second pumps may be operated substantially simultaneously.

Alternatively, the reagent may be added after an analysis. In some instances, a particle may interact with an analyte such that a change in the receptors attached to the first particle occurs. This change may not, however produce a detectable signal. The reagent attached to the second bead may be used to produce a detectable signal when it interacts with the first particle, if a specific analyte is present. In this embodiment, the fluid is introduced into the cavity first. After the analyte has been given time to react with the particle, the reagent may be added to the first cavity. The interaction of the reagent with the particle may produce a detectable signal. For example, an indicator reagent may react with a particle which has been exposed to an analyte to produce a color change on the particle. Particle which have not been exposed to the analyte may remain unchanged or show a different color change.

As shown in FIG. 1, a system for detecting analytes in a fluid may include a light source 110, a sensor array 120 and a detector 130. The sensor array 120 is preferably formed of a supporting member which is configured to hold a variety of particles 124 in an ordered array. A high sensitivity CCD array may be used to measure changes in optical characteristics which occur upon binding of the biological/chemical agents. Data acquisition and handling is preferably performed with existing CCD technology. As described above, colorimetric analysis may be performed using a white light source and a color CCD detector. However, color CCD detectors are typically more expensive than gray scale CCD detectors.

In one embodiment, a gray scale CCD detector may be used to detect colorimetric changes. In one embodiment, a gray scale detector may be disposed below a sensor array to measure the intensity of light being transmitted through the sensor array. A series of lights (e.g., light emitting diodes) may be arranged above the sensor array. In one embodiment, groups of three LED lights may be arranged above each of the cavities of the array. Each of these groups of LED lights may include a red, blue and a green light. Each of the lights may be operated individually such that one of the lights may be on while the other two lights are off. In order to provide color information while using a gray scale detector, each of the lights is sequentially turned on and the gray scale detector is used to measure the intensity of the light passing through the sensor array. After information from each of the lights is collected, the information may be processed to derive the absorption changes of the particle.

In one embodiment, the data collected by the gray scale detector may be recorded using 8 bits of data. Thus, the data will appear as a value between 0 and 255. The color of each chemical sensitive element may be represented as a red, blue and green value. For example, a blank particle (i.e., a particle which does not include a receptor) will typically appear white. When each of the LED lights (red, blue and green) are operated the CCD detector will record a value corresponding to the amount of light transmitted through the cavity. The intensity of the light may be compared to a blank particle, to determine the absorbance of a particle with respect to the LED light which is used. Thus, the red, green and blue components may be recorded individually without the use of a

color CCD detector. In one embodiment, it is found that a blank particle exhibits an absorbance of about 253 when illuminated with a red LED, a value of about 250 when illuminated by a green LED, and a value of about 222 when illuminated with a blue LED. This signifies that a blank particle does not significantly absorb red, green or blue light. When a particle with a receptor is scanned, the particle may exhibit a color change, due to absorbance by the receptor. For example, it was found that when a particle which includes a 5-carboxyfluorescein receptor is subjected to white light, the particle shows a strong absorbance of blue light. When a red LED is used to illuminate the particle, the gray scale CCD detector may detect a value of about 254. When the green LED is used, the gray scale detector may detect a value of about 218. When a blue LED light is used, a gray scale detector may detect a value of about 57. The decrease in transmittance of blue light is believed to be due to the absorbance of blue light by the 5-carboxyfluorescein. In this manner the color changes of a particle may be quantitatively characterized using a gray scale detector.

As described above, after the cavities are formed in the supporting member, a particle may be positioned at the bottom of a cavity using a micromanipulator. This allows the location of a particular particle to be precisely controlled during the production of the array. The use of a micromanipulator may, however, be impractical for production of sensor array systems. An alternate method of placing the particles into the cavities may involve the use of a silk screen like process. A series of masking materials may be placed on the upper surface of the sensor array prior to filling the cavities. The masking materials may be composed of glass, metal or plastic materials. A collection of particles may be placed upon the upper surface of the masking materials and the particles may be moved across the surface. When a cavity is encountered, a particle may drop into the cavity if the cavity is unmasked. Thus particles of known composition are placed in only the unmasked regions. After the unmasked cavities are filled, the masking pattern may be altered and a second type of particles may be spread across the surface. Preferably, the masking material will mask the cavities that have already been filled with particle. The masking material may also mask other non-filled cavities. This technique may be repeated until all of the cavities are filled. After filling the cavities, a cover may be placed on the support member, as described above, to inhibit the displacement and mixing of the particles. An advantage of such a process is that it may be more amenable to industrial production of supporting members.

Further modifications and alternative embodiments of various aspects of the invention will be apparent to those skilled in the art in view of this description. Accordingly, this description is to be construed as illustrative only and is for the purpose of teaching those skilled in the art the general manner of carrying out the invention. It is to be understood that the forms of the invention shown and described herein are to be taken as the presently preferred embodiments. Elements and materials may be substituted for those illustrated and described herein, parts and processes may be reversed, and certain features of the invention may be utilized independently, all as would be apparent to one skilled in the art after having the benefit of this description of the invention. Changes may be made in the elements described herein without departing from the spirit and scope of the invention as described in the following claims.

What is claimed is:

1. A system for detecting an analyte in a fluid comprising:  
a light source;  
5 a sensor array, the sensor array comprising a supporting member comprising at least one cavity formed within the supporting member;  
a particle, the particle positioned within the cavity, wherein the particle is configured to produce a signal when the particle interacts with the analyte during use; and  
a detector, the detector being configured to detect the signal produced by the interaction of the analyte  
10 with the particle during use;  
wherein the light source and detector are positioned such that light passes from the light source, to the particle, and onto the detector during use.
2. The system of claim 1, wherein the system comprises a plurality of particles positioned within a  
15 plurality of cavities, and wherein the system is configured to substantially simultaneously detect a plurality of analytes in the fluid.
3. The system of claim 1, wherein the system comprises a plurality of particles positioned within the cavity.
- 20 4. The system of claim 1, wherein the light source comprises a light emitting diode.
5. The system of claim 1, wherein the light source comprises a white light source.
- 25 6. The system of claim 1, wherein the sensor array further comprises a bottom layer and a top cover layer, wherein the bottom layer is positioned below a bottom surface of the supporting member, and wherein the top cover layer is positioned above the upper surface of the supporting member, and wherein the bottom layer and the top cover layer are positioned such that the particle is substantially contained within the cavity by the bottom layer and the top cover layer.
- 30 7. The system of claim 6, wherein the bottom layer and the top cover layer are substantially transparent to light produced by the light source.
8. The system of claim 1, wherein the sensor array further comprises a bottom layer and a top cover layer,  
35 wherein the bottom layer is coupled to a bottom surface of the supporting member, and wherein the top cover layer is coupled to a top surface of the supporting member; and wherein both the bottom layer and the top cover layer are coupled to the supporting member such that the particle is substantially contained within the cavity by bottom layer and the top cover layer.

9. The system of claim 8, wherein the bottom layer and the top cover layer are substantially transparent to light produced by the light source.
10. The system of claim 1, wherein the sensor array further comprises a bottom layer coupled to the supporting member, and wherein the supporting member comprises silicon, and wherein the bottom layer comprises silicon nitride.
11. The system of claim 1, wherein the sensor array further comprises a sensing cavity formed on a bottom surface of the sensor array.
12. The system of claim 1, wherein the supporting member is formed from a plastic material, and wherein the sensor array further comprises a top cover layer, the top cover layer being coupled to the supporting member such that the particle is substantially contained within the cavity, and wherein the top cover layer is configured to allow the fluid to pass through the top cover layer to the particle, and wherein both the supporting member and the top cover layer are substantially transparent to light produced by the light source.
13. The system of claim 1, further comprising a fluid delivery system coupled to the supporting member.
14. The system of claim 1, wherein the detector comprises a charge-coupled device.
15. The system of claim 1, wherein the detector comprises an ultraviolet detector.
16. The system of claim 1, wherein the detector comprises a fluorescence detector.
17. The system of claim 1, wherein the detector comprises a semiconductor based photodetector, and wherein the detector is coupled to the sensor array.
18. The system of claim 1, wherein the particle ranges from about 0.05 micron to about 500 microns.
19. The system of claim 1, wherein a volume of the particle changes when contacted with the fluid.
20. The system of claim 1, wherein the particle comprises a metal oxide particle.
21. The system of claim 1, wherein the particle comprises a metal quantum particle.
22. The system of claim 1, wherein the particle comprises a semiconductor quantum particle.

23. The system of claim 1, wherein the particle comprises a receptor molecule coupled to a polymeric resin.
24. The system of claim 23, wherein the polymeric resin comprises polystyrene-polyethylene glycol-divinyl benzene.
25. The system of claim 23, wherein the receptor molecule produces the signal in response to the pH of the fluid.
26. The system of claim 23, wherein the analyte comprises a metal ion, and wherein the receptor produces the signal in response to the presence of the metal ion.
27. The system of claim 23, wherein the analyte comprises a carbohydrate, and wherein the receptor produces a signal in response to the presence of a carbohydrate.
28. The system of claim 23, wherein the particles further comprises a first indicator and a second indicator, the first and second indicators being coupled to the receptor, wherein the interaction of the receptor with the analyte causes the first and second indicators to interact such that the signal is produced.
29. The system of claim 23, wherein the particles further comprises an indicator, wherein the indicator is associated with the receptor such that in the presence of the analyte the indicator is displaced from the receptor to produce the signal.
30. The system of claim 23, wherein the receptor comprises a polynucleotide.
31. The system of claim 23, wherein the receptor comprises a peptide.
32. The system of claim 23, wherein the receptor comprises an enzyme.
33. The system of claim 23, wherein the receptor comprises a synthetic receptor.
34. The system of claim 23, wherein the receptor comprises an unnatural biopolymer.
35. The system of claim 23, wherein the receptor comprises an antibody.
36. The system of claim 23, wherein the receptor comprises an antigen.
37. The system of claim 1, wherein the analyte comprises phosphate functional groups, and wherein the particle is configured to produce the signal in the presence of the phosphate functional groups.

38. The system of claim 1, wherein the analyte comprises bacteria, and wherein the particle is configured to produce the signal in the presence of the bacteria.
39. The system of claim 1, wherein the system comprises a plurality of particles positioned within a plurality of cavities, and wherein the plurality of particles produce a detectable pattern in the presence of the analyte.
40. A system for detecting an analyte in a fluid comprising:  
a light source;  
a sensor array, the sensor array comprising a supporting member comprising a plurality of cavities formed within the supporting member, wherein the supporting member comprises silicon;  
a plurality of particles, the particles comprising a receptor molecule covalently linked to a polymeric resin, wherein the particles are positioned within the cavities, and wherein each of the particles is configured to produce a signal when the particle interacts with the analyte during use; and  
a detector configured to detect the signal produced by the interaction of the analyte with the particle during use;  
wherein the light source and detector are positioned such that light passes from the light source, to the particle, and onto the detector during use.
41. The system of claim 40, wherein the system is configured to substantially simultaneously detect a plurality of analytes in the fluid.
42. The system of claim 40, wherein each cavity is configured to hold a single particle.
43. The system of claim 40, wherein each cavity is configured to hold a plurality of particles.
44. The system of claim 40, wherein the sensor array further comprises a bottom layer and a top cover layer, wherein the bottom layer is positioned below a bottom surface of the supporting member, and wherein the top cover layer is positioned above the upper surface of the supporting member, and wherein the bottom layer and the top cover layer are positioned such that the particle is substantially contained within the cavity by the bottom layer and the top cover layer.
45. The system of claim 44, wherein the bottom layer and the top cover layer are substantially transparent to light produced by the light source.
46. The system of claim 40, wherein the sensor array further comprises a bottom layer and a top cover layer, wherein the bottom layer is coupled to a bottom surface of the supporting member, and wherein the top cover layer is coupled to a top surface of the supporting member; and wherein both the bottom layer and the top cover layer are coupled to the supporting member such that the particle is



substantially contained within the cavity by bottom layer and the top cover layer.

47. The system of claim 46, wherein the bottom layer and the top cover layer are substantially transparent to light produced by the light source.
- 5
48. The system of claim 40, wherein the sensor array further comprises a bottom layer coupled to the supporting member, and wherein the bottom layer comprises silicon nitride.
49. The system of claim 40, wherein the sensor array further comprises a sensing cavity formed on a bottom surface of the sensor array.
- 10
50. The system of claim 40, further comprising a fluid delivery system coupled to the supporting member.
51. The system of claim 40, wherein the detector comprises a charge-coupled device.
- 15
52. The system of claim 40, wherein the detector comprises a semiconductor based photodetector, and wherein the detector is coupled to the sensor array.
53. The system of claim 40, wherein the particle ranges from about 0.05 micron to about 500 microns.
- 20
54. The system of claim 40, wherein a volume of the particle changes when contacted with the fluid.
55. The system of claim 40, wherein the polymeric bead comprises a polystyrene-polyethylene glycol-divinyl benzene resin.
- 25
56. The system of claim 40, wherein the receptor molecule produces the signal in response to the pH of the fluid.
57. The system of claim 40, wherein the analyte comprises a metal ion, and wherein the receptor produces the signal in response to the presence of the metal ion.
- 30
58. The system of claim 40, wherein the analyte comprises a carbohydrate, and wherein the receptor produces a signal in response to the presence of the carbohydrate.
59. The system of claim 40, wherein the particles further comprises a first indicator and a second indicator, the first and second indicators being coupled to the receptor, wherein the interaction of the receptor with the analyte causes the first and second indicators to interact such that the signal is produced.
- 35
60. The system of claim 40, wherein the particles further comprises an indicator, wherein the indicator is

associated with the receptor such that in the presence of the analyte the indicator is displaced from the receptor to produce the signal.

- 5
61. The system of claim 40, wherein the receptor comprises a polynucleotide.
62. The system of claim 40, wherein the receptor comprises a peptide.
63. The system of claim 40, wherein the receptor comprises an enzyme.
- 10 64. The system of claim 40, wherein the receptor comprises a synthetic receptor.
65. The system of claim 40, wherein the receptor comprises an unnatural biopolymer.
66. The system of claim 40, wherein the receptor comprises an antibody.
- 15 67. The system of claim 40, wherein the receptor comprises an antigen.
68. The system of claim 40, wherein the particles produce a detectable pattern in the presence of the analyte.
- 20 69. A sensor array for detecting an analyte in a fluid comprising:  
a supporting member; wherein at least one cavity is formed within the supporting member;  
a particle positioned within the cavity, wherein the particle is configured to produce a signal when the  
particle interacts with the analyte.
- 25 70. The sensor array of claim 69, further comprising a plurality of particles positioned within the cavity.
71. The sensor array of claim 69, wherein the particle comprises a receptor molecule coupled to a  
polymeric resin.
- 30 72. The sensor array of claim 69, wherein the particle has a size ranging from about 0.05 micron to about  
500 microns in diameter.
73. The sensor array of claim 69, wherein the particle has a size ranging from about 0.05 micron to about  
35 500 microns in diameter, and wherein the cavity is configured to substantially contain the particle.
74. The sensor array of claim 69, wherein the supporting member comprises a plastic material.
75. The sensor array of claim 69, wherein the supporting member comprises a silicon wafer.

76. The sensor array of claim 75, wherein the cavity extends through the silicon wafer.
77. The sensor array of claim 75, wherein the cavity is substantially pyramidal in shape and wherein the sidewalls of the cavity are substantially tapered at an angle of between about 50 to about 60 degrees.
- 5 78. The sensor array of claim 75, further comprising a substantially transparent layer positioned on a bottom surface of the silicon wafer.
- 10 79. The sensor array of claim 75, further comprising a substantially transparent layer positioned on a bottom surface of the silicon wafer, wherein the substantially transparent layer comprises silicon dioxide, silicon nitride, or silicon oxide/silicon nitride multilayer stacks.
- 15 80. The sensor array of claim 75, further comprising a substantially transparent layer positioned on a bottom surface of the silicon wafer, wherein the substantially transparent layer comprises silicon nitride.
81. The sensor array of claim 75, wherein the silicon wafer has an area of about 1 cm<sup>2</sup> to about 100 cm<sup>2</sup>.
- 20 82. The sensor array of claim 75, further comprising a plurality of cavities formed in the silicon wafer, and wherein from about 10 to about 10<sup>6</sup> cavities are formed in the silicon wafer.
83. The sensor array of claim 69, further comprising channels in the supporting member, wherein the channels are configured to allow the fluid to flow through the channels into and away from the cavity.
- 25 84. The sensor array of claim 69, further comprising an inner surface coating, wherein the inner surface coating is configured to inhibit dislodgment of the particle.
85. The sensor array of claim 69, further comprising a detector coupled to the bottom surface of the supporting member, wherein the detector is positioned below the cavity.
- 30 86. The sensor array of claim 85, wherein the detector is a semiconductor based photodetector.
87. The sensor array of claim 85, wherein the detector is a Fabry-Perot type detector.
- 35 88. The sensor array of claim 85, further comprising an optical fiber coupled to the detector, wherein the optical fiber is configured to transmit optical data to a microprocessor.
89. The sensor array of claim 69, further comprising an optical filters coupled to a bottom surface of the sensor array.

90. The sensor array of claim 69, further comprising a barrier layer positioned over the cavity, the barrier layer being configured to inhibit dislodgment of the particle during use.
- 5 91. The sensor array of claim 90, wherein the barrier layer comprises a substantially transparent cover plate positioned over the cavity, and wherein the cover plate is positioned a fixed distance over the cavity such that the fluid can enter the cavity.
92. The sensor array of claim 91, wherein the barrier layer comprises plastic, glass, quartz, silicon oxide, or silicon nitride.
- 10 93. The sensor array of claim 69, further comprising a plurality of particles positioned within a plurality of cavities formed in the supporting member.
94. The sensor array of claim 69, wherein the system comprises a plurality of particles positioned within a plurality of cavities, and wherein the plurality of particles produce a detectable pattern in the presence of the analyte.
- 15 95. A sensor array for detecting an analyte in a fluid comprising:  
a supporting member, wherein the supporting member comprises a silicon wafer, and wherein a plurality of cavities are formed within the supporting member;  
20 a plurality of particles, at least one particle being positioned in each of the cavities, wherein the particles are configured to produce a signal when the particles interact with the analyte.
96. The sensor array of claim 95, wherein a plurality of particles is positioned within each of the cavities.
- 25 97. The sensor array of claim 95, wherein the particles comprise a receptor molecule coupled to a polymeric bead.
98. The sensor array of claim 95, wherein the cavity extends through the supporting member.
- 30 99. The sensor array of claim 95, wherein the cavity is substantially pyramidal in shape and wherein the sidewalls of the cavity are substantially tapered at an angle of between about 50 to about 60 degrees.
100. The sensor array of claim 95, further comprising a substantially transparent layer positioned on a bottom surface of the supporting member.
- 35 101. The sensor array of claim 95, further comprising a substantially transparent layer positioned on a bottom surface of the silicon wafer, wherein the substantially transparent layer comprises silicon dioxide, silicon nitride, or silicon oxide/silicon nitride multilayer stacks.

102. The sensor array of claim 95, further comprising a substantially transparent layer positioned on a bottom surface of the silicon wafer, wherein the substantially transparent layer comprises silicon nitride.
- 5 103. The sensor array of claim 95, wherein the silicon wafer has an area of about 1 cm<sup>2</sup> to about 100 cm<sup>2</sup>.
104. The sensor array of claim 95, further comprising a plurality of cavities formed in the silicon wafer, and wherein from about 10 to about 10<sup>6</sup> cavities are formed in the silicon wafer.
- 10 105. The sensor array of claim 95, further comprising channels in the supporting member, wherein the channels are configured to allow the fluid to flow through the channels into and away from the cavities.
106. The sensor array of claim 95, further comprising an inner surface coating, wherein the inner surface  
15 coating is configured to inhibit dislodgment of the particle.
107. The sensor array of claim 95, further comprising a detector coupled to the bottom surface of the supporting member, wherein the detector is positioned below the cavity.
- 20 108. The sensor array of claim 107, wherein the detector is a semiconductor based photodetector.
109. The sensor array of claim 107, wherein the detector is a Fabry-Perot type detector.
110. The sensor array of claim 107, further comprising an optical fiber coupled to the detector, wherein the  
25 optical fiber is configured to transmit optical data to a microprocessor.
111. The sensor array of claim 95, further comprising a barrier layer positioned over the cavity, the barrier layer being configured to inhibit dislodgment of the particle during use.
- 30 112. The sensor array of claim 111, wherein the barrier layer comprises a cover plate positioned over the cavity, and wherein the cover plate is positioned a fixed distance over the cavity such that the fluid can enter the cavity.
113. The sensor array of claim 111, further comprising channels in the supporting member, wherein the  
35 channels are configured to allow the fluid to flow through the channels into and away from the cavities, and wherein the barrier layer comprises a cover plate positioned upon an upper surface of the supporting member, and wherein the cover plate inhibits passage of the fluid into the cavities such that the fluid enters the cavities via the channels.

114. The sensor array of claim 111, wherein the barrier layer comprises plastic, glass, quartz, silicon oxide, or silicon nitride.
115. A method for forming a sensor array configured to detect an analyte in a fluid, comprising:  
5 forming a cavity in a supporting member, wherein the supporting member comprises a silicon wafer;  
placing a particle in the cavity, wherein the particle is configured to produce a signal when the particle interacts with the analyte; and  
forming a cover upon a portion of the supporting member, wherein the cover is configured to inhibit dislodgment of the particle from the cavity.
- 10 116. The method of claim 115, wherein forming the cavity comprises anisotropically etching the silicon wafer.
117. The method of claim 115, wherein forming the cavity comprises anisotropically etching the silicon  
15 wafer with a wet hydroxide etch.
118. The method of claim 115, wherein forming the cavity comprises anisotropically etching the silicon wafer such that sidewalls of the cavity are tapered at an angle from about 50 degrees to about 60 degrees.
- 20 119. The method of claim 115, wherein the silicon wafer has an area of about 1 cm<sup>2</sup> to about 100 cm<sup>2</sup>.
120. The method of claim 115, further comprising forming a substantially transparent layer upon a bottom surface of the silicon wafer below the cavity.
- 25 121. The method of claim 115, further comprising forming a substantially transparent layer upon a bottom surface of the silicon wafer, wherein the cavity extends through the silicon wafer and wherein the substantially transparent layer is positioned to support the particle.
- 30 122. The method of claim 115, wherein the substantially transparent layer comprises silicon nitride.
123. The method of claim 115, wherein the cover comprises plastic, glass, quartz, silicon nitride, or silicon oxide.
- 35 124. The method of claim 115, wherein forming the cover comprises coupling the cover to the silicon wafer at a distance above the silicon wafer substantially less than a width of the particle.
125. The method of claim 115, further comprising etching channels in the silicon wafer prior to forming the cover on the silicon wafer, wherein forming the cover comprises placing the cover against the upper

surface of the silicon wafer, and wherein the channels are configured to allow the fluid to pass through the silicon wafer to and from the cavities.

- 5 126. The method of claim 115, further comprising coating an inner surface of the cavity with a material to increase adhesion of the particle to the inner surface of the cavity.
127. The method of claim 115, further comprising coating an inner surface of the cavity with a material to increase reflectivity of the inner surface of the cavity.
- 10 128. The method of claim 115, further comprising forming an optical detector upon a bottom surface of the supporting member below the cavity.
129. The method of claim 115, further comprising forming a sensing cavity upon a bottom surface of the supporting member below the cavity.
- 15 130. The method of claim 129, wherein forming the sensing cavity comprises:  
forming a barrier layer upon a bottom surface of the silicon wafer;  
forming a bottom diaphragm layer upon the barrier layer;  
forming etch windows extending through the bottom diaphragm layer;  
20 forming a sacrificial spacer layer upon the bottom diaphragm layer;  
removing a portion of the spacer layer;  
forming a top diaphragm layer; and  
removing a remaining portion of the spacer layer.
- 25 131. The method of claim 130, further comprising filling a portion of the sensing cavity with a sensing substrate.
132. The method of claim 115, further comprising forming an optical filter upon the bottom surface of the supporting member.
- 30 133. The method of claim 115, further comprising forming a plurality of cavities in the silicon wafer.
134. The method of claim 115, wherein from about 10 to about  $10^6$  cavities are formed in the silicon wafer.
- 35 135. A sensor array produced by the method of claim 115.
136. A method of sensing an analyte in a fluid comprising:  
passing a fluid over a sensor array, the sensor array comprising at least one particle positioned within a cavity of a supporting member;

monitoring a spectroscopic change of the particle as the fluid is passed over the sensor array, wherein the spectroscopic change is caused by the interaction of the analyte with the particle.

- 5      137.      The method of claim 136, wherein the spectroscopic change comprises a change in absorbance of the particle.
138.      The method of claim 136, wherein the spectroscopic change comprises a change in fluorescence of the particle.
- 10      139.      The method of claim 136, wherein the spectroscopic change comprises a change in phosphorescence of the particle.
140.      The method of claim 136, wherein the analyte is a proton atom, and wherein the spectroscopic change is produced when the pH of the fluid is varied, and wherein monitoring the spectroscopic change of the particle allows the pH of the fluid to be determined.
- 15      141.      The method of claim 136, wherein the analyte is a metal cation, and wherein the spectroscopic change is produced in response to the presence of the metal cation in the fluid.
- 20      142.      The method of claim 136, wherein the analyte is an anion, and wherein the spectroscopic change is produced in response to the presence of the anion in the fluid.
143.      The method of claim 136, wherein the analyte is a DNA molecule, and wherein the spectroscopic change is produced in response to the presence of the DNA molecule in the fluid.
- 25      144.      The method of claim 136, wherein the analyte is a protein, and wherein the spectroscopic change is produced in response to the presence of the protein in the fluid.
145.      The method of claim 136, wherein the analyte is a metabolite, and wherein the spectroscopic change is produced in response to the presence of the metabolite in the fluid.
- 30      146.      The method of claim 136, wherein the analyte is a sugar, and wherein the spectroscopic change is produced in response to the presence of the sugar in the fluid.
147.      The method of claim 136, wherein the analyte is a bacteria, and wherein the spectroscopic change is produced in response to the presence of the bacteria in the fluid.
- 35      148.      The method of claim 136, wherein the particle comprises a receptor coupled to a polymeric resin, and further comprising exposing the particle to an indicator prior to passing the fluid over the sensor array.



149. The method of claim 148, wherein a binding strength of the indicator to the receptor is less than a binding strength of the analyte to the receptor.
150. The method of claim 148, wherein the indicator is a fluorescent indicator.
- 5 151. The method of claim 136, further comprising treating the fluid with an indicator prior to passing the fluid over the sensor array, wherein the indicator is configured to couple with the analyte.
- 10 152. The method of claim 136, wherein the analyte is bacteria and further comprising breaking down the bacteria prior to passing the fluid over the sensor array.
153. The method of claim 136, wherein monitoring the spectroscopic change is performed with a CCD device.
- 15 154. The method of claim 136, further comprising measuring the intensity of the spectroscopic change, and further comprising calculating the concentration of the analyte based on the intensity of the spectroscopic change.
- 20 155. A sensor array for detecting an analyte in a fluid comprising:  
a supporting member, wherein the supporting member comprises a silicon wafer, and wherein a plurality of cavities are formed within the supporting member;  
a plurality of particles, at least one particle being positioned in each of the cavities wherein the particles are configured to produce a signal when the particles interact with the analyte.
- 25 156. A method of sensing an analyte in a fluid comprising:  
passing a fluid over a sensor array, the sensor array comprising:  
a supporting member, wherein the supporting member comprises a silicon wafer, and wherein  
a plurality of cavities are formed within the supporting member; and  
a plurality of particles, at least one particle being positioned in each of the cavities, wherein  
30 the particles are configured to produce a signal when the particles interact with the analyte at least one particle positioned within a cavity of a supporting member;  
monitoring a spectroscopic change of the particle as the fluid is passed over the sensor array, wherein the spectroscopic change is caused by the interaction of the analyte with the particle.
- 35 157. The method of claim 156, wherein the spectroscopic change comprises a change in absorbance of the particle.
158. The method of claim 156, wherein the spectroscopic change comprises a change in reflectance of the particle.

159. The method of claim 156, wherein the spectroscopic change comprises a change in fluorescence of the particle.
160. The method of claim 156, wherein the spectroscopic change comprises a change in phosphorescence of the particle.
161. The method of claim 156, wherein the analyte is a proton atom, and wherein the spectroscopic change is produced when the pH of the fluid is varied, and wherein monitoring the spectroscopic change of the particle allows the pH of the fluid to be determined.
162. The method of claim 156, wherein the analyte is a metal cation, and wherein the spectroscopic change is produced in response to the presence of the metal cation in the fluid.
163. The method of claim 156, wherein the particle comprises a receptor coupled to a polymeric resin, and further comprising exposing the particle to an indicator prior to passing the fluid over the sensor array.
164. The method of claim 156, wherein a binding strength of the indicator to the receptor is less than a binding strength of the analyte to the receptor.
165. The method of claim 156, wherein the indicator is a fluorescent indicator.
166. The method of claim 156, further comprising treating the fluid with an indicator prior to passing the fluid over the sensor array, wherein the indicator is configured to couple with the analyte.
167. The method of claim 156, wherein the analyte is bacteria and further comprising breaking down the bacteria prior to passing the fluid over the sensor array.
168. The method of claim 156, wherein monitoring the spectroscopic change is performed with a CCD device.
169. The method of claim 156, further comprising measuring the intensity of the spectroscopic change, and further comprising calculating the concentration of the analyte based on the intensity of the spectroscopic change.
170. A system for detecting an analyte in a fluid comprising:  
a light source;  
a sensor array, the sensor array comprising at least one particle coupled to the sensor array, wherein the particle is configured to produce a signal when the particle interacts with the analyte; and  
a detector configured to detect the signal produced by the interaction of the analyte with the particle;

wherein the light source and detector are positioned such that light passes from the light source, to the particle, and onto the detector during use.

171. A sensor array for detecting an analyte in a fluid comprising:  
5           at least one particle coupled to the sensor array, wherein the particle is configured to produce a signal when the particle interacts with the analyte.
172. A method of sensing an analyte in a fluid comprising:  
10           passing a fluid over a sensor array, the sensor array comprising at least one particle coupled to a supporting member;  
            monitoring a spectroscopic change of the particle as the fluid is passed over the sensor array, wherein the spectroscopic change is caused by the interaction of the analyte with the particle.
173. A sensor array for detecting an analyte in a fluid comprising:  
15           a supporting member; wherein at least one cavity is formed within the supporting member;  
            a particle positioned within the cavity, wherein the particle is configured to produce a signal when the particle interacts with the analyte;  
            wherein the cavities are configured to allow the fluid to pass through the supporting member during use.
- 20   174. The sensor array of claim 173, further comprising a plurality of particles positioned within the cavity.
175. The sensor array of claim 173, wherein the particle comprises a receptor molecule coupled to a polymeric resin.
- 25   176. The sensor array of claim 173, wherein the particle has a size ranging from about 0.05 micron to about 500 microns in diameter.
177. The sensor array of claim 173, wherein the cavity is configured to substantially contain the particle.
- 30   178. The sensor array of claim 173, further comprising a cover layer coupled to the supporting member and a bottom layer coupled to the supporting member, wherein the cover layer and the bottom layer are removable.
- 35   179. The sensor array of claim 173, further comprising a cover layer coupled to the supporting member and a bottom layer coupled to the supporting member, wherein the cover layer and the bottom layer are removable, and wherein the cover layer and the bottom layer include openings that are substantially aligned with the cavities during use.
180. The sensor array of claim 173, further comprising a cover layer coupled to the supporting member and

a bottom layer coupled to the supporting member, wherein the bottom layer is coupled to a bottom surface of the supporting member and wherein the cover layer is removable. and wherein the cover layer and the bottom layer include openings that are substantially aligned with the cavities during use.

- 5     181.     The sensor array of claim 173, further comprising a cover layer coupled to the supporting member and a bottom layer coupled to the supporting member, wherein an opening is formed in the cover layer substantially aligned with the cavity, and wherein an opening is formed in the bottom layer substantially aligned with the cavity.
- 10    182.     The sensor array of claim 173, wherein the cavity is substantially tapered such that the width of the cavity narrows in a direction from a top surface of the supporting member toward a bottom surface of the supporting member, and wherein a minimum width of the cavity is substantially less than a width of the particle.
- 15    183.     The sensor array of claim 173, wherein a width of a bottom portion of the cavity is substantially less than a width of a top portion of the cavity, and wherein the width of the bottom portion of the cavity is substantially less than a width of the particle.
- 20    184.     The sensor array of claim 173, further comprising a cover layer coupled to the supporting member and a bottom layer coupled to the supporting member, wherein the bottom layer is configured to support the particle, and wherein an opening is formed in the cover layer substantially aligned with the cavity.
- 25    185.     The sensor array of claim 173, further comprising a removable cover layer coupled to the supporting member.
186.     The sensor array of claim 173, wherein the supporting member comprises a plastic material.
187.     The sensor array of claim 173, wherein the supporting member comprises a silicon wafer.
- 30    188.     The sensor array of claim 173, wherein the supporting member comprises a dry film photoresist material.
189.     The sensor array of claim 173, wherein the supporting member comprises a plurality of layers of a dry film photoresist material.
- 35    190.     The sensor array of claim 173, wherein an inner surface of the cavity is coated with a reflective material.
191.     The sensor array of claim 173, further comprising channels in the supporting member, wherein the

channels are configured to allow the fluid to flow through the channels into and away from the cavity.

192. The sensor array of claim 173, further comprising a plurality of additional particles positioned within a plurality of additional cavities formed in the supporting member.

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193. A system for detecting an analyte in a fluid comprising:  
a light source;  
a sensor array, the sensor array comprising a supporting member comprising at least one cavity formed within the supporting member, wherein the cavity is configured such that the fluid entering the cavity passes through the supporting member during use;  
a particle, the particle positioned within the cavity, wherein the particle is configured to produce a signal when the particle interacts with the analyte during use; and  
a detector, the detector being configured to detect the signal produced by the interaction of the analyte with the particle during use;  
wherein the light source and detector are positioned such that light passes from the light source, to the particle, and onto the detector during use.

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194. The system of claim 193, wherein the system comprises a plurality of particles positioned within a plurality of cavities, and wherein the system is configured to substantially simultaneously detect a plurality of analytes in the fluid.

195. The system of claim 193, wherein the system comprises a plurality of particles positioned within the cavity.

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196. The system of claim 193, wherein the light source comprises a light emitting diode.

197. The system of claim 193, wherein the light source comprises a red light emitting diode, a blue light emitting diode, and a green light emitting diode.

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198. The system of claim 193, wherein the light source comprises a white light source.

199. The system of claim 193, wherein the sensor array further comprises a cover layer coupled to the supporting member and a bottom layer coupled to the supporting member, wherein the cover layer and the bottom layer are removable.

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200. The system of claim 193, wherein the sensor array further comprises a cover layer coupled to the supporting member and a bottom layer coupled to the supporting member, wherein the cover layer and the bottom layer are removable, and wherein the cover layer and the bottom layer include openings that are substantially aligned with the cavities during use.

201. The system of claim 193, wherein the sensor array further comprises a cover layer coupled to the supporting member and a bottom layer coupled to the supporting member, wherein the bottom layer is coupled to a bottom surface of the supporting member and wherein the cover layer is removable, and  
5 wherein the cover layer and the bottom layer include openings that are substantially aligned with the cavities during use.
202. The system of claim 193, wherein the sensor array further comprises a cover layer coupled to the supporting member and a bottom layer coupled to the supporting member, wherein an opening is  
10 formed in the cover layer substantially aligned with the cavity, and wherein an opening is formed in the bottom layer substantially aligned with the cavity.
203. The system of claim 193, wherein the cavity is substantially tapered such that the width of the cavity narrows in a direction from a top surface of the supporting member toward a bottom surface of the supporting member, and wherein a minimum width of the cavity is substantially less than a width of  
15 the particle.
204. The system of claim 193, wherein a width of a bottom portion of the cavity is substantially less than a width of a top portion of the cavity, and wherein the width of the bottom portion of the cavity is  
20 substantially less than a width of the particle.
205. The system of claim 193, wherein the sensor array further comprises a cover layer coupled to the supporting member and a bottom layer coupled to the supporting member, wherein the bottom layer is configured to support the particle, and wherein an opening is formed in the cover layer substantially  
25 aligned with the cavity.
206. The system of claim 193, further comprising a removable cover layer.
207. The system of claim 193, wherein the supporting member comprises a plastic material.  
30
208. The system of claim 193, wherein the supporting member comprises a silicon wafer.
209. The system of claim 193, wherein the supporting member comprises a dry film photoresist material.
- 35 210. The system of claim 193, wherein the supporting member comprises a plurality of layers of a dry film photoresist material.
211. The system of claim 193, wherein an inner surface of the cavity is coated with a reflective material.
212. The system of claim 193, further comprising channels in the supporting member, wherein the channels

are configured to allow the fluid to flow through the channels into and away from the cavity.

213. The system of claim 193, wherein the detector comprises a charge-coupled device.

5 214. The system of claim 193, wherein the detector comprises a semiconductor based photodetector, and wherein the detector is coupled to the sensor array.

215. The system of claim 193, wherein the particle comprises a receptor molecule coupled to a polymeric resin.

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216. The system of claim 215, wherein the polymeric resin comprises polystyrene-polyethylene glycol-divinyl benzene.

15

217. The system of claim 215, wherein the receptor molecule produces the signal in response to the pH of the fluid.

218. The system of claim 215, wherein the analyte comprises a metal ion, and wherein the receptor produces the signal in response to the presence of the metal ion.

20

219. The system of claim 215, wherein the analyte comprises a carbohydrate, and wherein the receptor produces a signal in response to the presence of a carbohydrate.

25

220. The system of claim 215, wherein the particles further comprises a first indicator and a second indicator, the first and second indicators being coupled to the receptor, wherein the interaction of the receptor with the analyte causes the first and second indicators to interact such that the signal is produced.

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221. The system of claim 215, wherein the particles further comprises an indicator, wherein the indicator is associated with the receptor such that in the presence of the analyte the indicator is displaced from the receptor to produce the signal.

222. The system of claim 215, wherein the receptor comprises a polynucleotide.

35

223. The system of claim 215, wherein the receptor comprises a peptide.

224. The system of claim 215, wherein the receptor comprises an enzyme.

225. The system of claim 215, wherein the receptor comprises a synthetic receptor.

226. The system of claim 215, wherein the receptor comprises an unnatural biopolymer.

227. The system of claim 215, wherein the receptor comprises an antibody.
228. The system of claim 215, wherein the receptor comprises an antigen.
- 5 229. The system of claim 193, wherein the analyte comprises phosphate functional groups, and wherein the particle is configured to produce the signal in the presence of the phosphate functional groups.
- 10 230. The system of claim 193, wherein the analyte comprises bacteria, and wherein the particle is configured to produce the signal in the presence of the bacteria.
231. The system of claim 193, wherein the system comprises a plurality of particles positioned within a plurality of cavities, and wherein the plurality of particles produce a detectable pattern in the presence of the analyte.
- 15 232. A sensor array for detecting an analyte in a fluid comprising:  
a supporting member; wherein at least one cavity is formed within the supporting member;  
a particle positioned within the cavity, wherein the particle is configured to produce a signal when the particle interacts with the analyte; and  
20 a pump coupled to the supporting member, wherein the pump is configured to direct the fluid towards the cavity;  
wherein a channel is formed in the supporting member, the channel coupling the pump to the cavity such that the fluid flows through the channel to the cavity during use.
- 25 233. The sensor array of claim 232, wherein the particle comprises a receptor molecule coupled to a polymeric resin.
234. The sensor array of claim 232, wherein the supporting member comprises a plastic material.
- 30 235. The sensor array of claim 232, wherein the supporting member comprises a silicon wafer.
236. The sensor array of claim 232, wherein the supporting member comprises a dry film photoresist material.
- 35 237. The sensor array of claim 232, wherein the supporting member comprises a plurality of layers of a dry film photoresist material.



238. The sensor array of claim 232, wherein an inner surface of the cavity is coated with a reflective material.
- 5 239. The sensor array of claim 232, further comprising a detector coupled to the bottom surface of the supporting member, wherein the detector is positioned below the cavity.
240. The sensor array of claim 232, further comprising a barrier layer positioned over the cavity, the barrier layer being configured to inhibit dislodgment of the particle during use.
- 10 241. The sensor array of claim 232, further comprising a barrier layer positioned over the cavity, the barrier layer being configured to inhibit dislodgment of the particle during use, wherein the barrier layer comprises a transmission electron microscope grid.
242. The sensor array of claim 232, further comprising a plurality of particles positioned within a plurality of cavities formed in the supporting member.
- 15 243. The sensor array of claim 232, wherein the system comprises a plurality of particles positioned within a plurality of cavities, and wherein the plurality of particles produce a detectable pattern in the presence of the analyte.
- 20 244. The sensor array of claim 232, wherein the pump comprises a diaphragm pump.
245. The sensor array of claim 232, wherein the pump comprises an electrode pump.
- 25 246. The sensor array of claim 232 wherein the pump comprises a piezoelectric pump.
247. The sensor array of claim 232, wherein the pump comprises a pneumatic activated pump.
248. The sensor array of claim 232, wherein the pump comprises a heat activated pump.
- 30 249. The sensor array of claim 232, wherein the pump comprises a peristaltic pump.
250. The sensor array of claim 232, wherein the pump comprises an electroosmosis pump.
- 35 251. The sensor array of claim 232, wherein the pump comprises an electrohydrodynamic pump.
252. The sensor array of claim 232, wherein the pump comprises an electroosmosis pump and an electrohydrodynamic pump.

253. The sensor array of claim 232, wherein the cavity is substantially tapered such that the width of the cavity narrows in a direction from a top surface of the supporting member toward a bottom surface of the supporting member, and wherein a minimum width of the cavity is substantially less than a width of the particle.
- 5 254. The sensor array of claim 232, wherein a width of a bottom portion of the cavity is substantially less than a width of a top portion of the cavity, and wherein the width of the bottom portion of the cavity is substantially less than a width of the particle.
- 10 255. A system for detecting an analyte in a fluid comprising:  
a light source;  
a sensor array, the sensor array comprising a supporting member comprising at least one cavity formed within the supporting member, a pump coupled to the supporting member, wherein the pump is configured to direct the fluid towards the cavity, and wherein a channel is formed in the supporting member, the channel coupling the pump to the cavity such that the fluid flows through the channel to the cavity during use;  
15 a particle, the particle positioned within the cavity, wherein the particle is configured to produce a signal when the particle interacts with the analyte during use; and  
a detector, the detector being configured to detect the signal produced by the interaction of the analyte with the particle during use;  
20 wherein the light source and detector are positioned such that light passes from the light source, to the particle, and onto the detector during use.
- 25 256. The system of claim 255, wherein the system comprises a plurality of particles positioned within a plurality of cavities, and wherein the system is configured to substantially simultaneously detect a plurality of analytes in the fluid.
257. The system of claim 255, wherein the light source comprises a light emitting diode.
- 30 258. The system of claim 255, wherein the light source comprises a red light emitting diode, a blue light emitting diode, and a green light emitting diode.
259. The system of claim 255, wherein the light source comprises a white light source.
- 35 260. The system of claim 255, wherein the supporting member comprises a plastic material.
261. The system of claim 255, wherein the supporting member comprises a silicon wafer.
262. The system of claim 255, wherein the supporting member comprises a dry film photoresist material.

263. The system of claim 255, wherein the supporting member comprises a plurality of layers of a dry film photoresist material.
264. The system of claim 255, wherein an inner surface of the cavity is coated with a reflective material.
- 5 265. The system of claim 255, further comprising a barrier layer coupled to the supporting member, wherein the barrier layer is positioned over the cavity, the barrier layer being configured to inhibit dislodgment of the particle during use.
- 10 266. The system of claim 255, wherein the pump comprises a diaphragm pump.
267. The system of claim 255, wherein the pump comprises an electrode pump.
268. The system of claim 255 wherein the pump comprises a piezoelectric pump.
- 15 269. The system of claim 255, wherein the pump comprises a pneumatic activated pump.
270. The system of claim 255, wherein the pump comprises a heat activated pump.
- 20 271. The system of claim 255, wherein the pump comprises a peristaltic pump.
272. The system of claim 255, wherein the pump comprises an electroosmosis pump.
273. The system of claim 255, wherein the pump comprises an electrohydrodynamic pump.
- 25 274. The system of claim 255, wherein the pump comprises an electroosmosis pump and an electrohydrodynamic pump.
- 30 275. The system of claim 255, wherein the cavity is substantially tapered such that the width of the cavity narrows in a direction from a top surface of the supporting member toward a bottom surface of the supporting member, and wherein a minimum width of the cavity is substantially less than a width of the particle.
- 35 276. The system of claim 255, wherein a width of a bottom portion of the cavity is substantially less than a width of a top portion of the cavity, and wherein the width of the bottom portion of the cavity is substantially less than a width of the particle.
277. The system of claim 255, wherein the detector comprises a charge-coupled device.

278. The system of claim 255, wherein the particle comprises a receptor molecule coupled to a polymeric resin.
279. The system of claim 278, wherein the polymeric resin comprises polystyrene-polyethylene glycol-divinyl benzene.
280. The system of claim 278, wherein the particles further comprises a first indicator and a second indicator, the first and second indicators being coupled to the receptor, wherein the interaction of the receptor with the analyte causes the first and second indicators to interact such that the signal is produced.
281. The system of claim 278, wherein the particles further comprises an indicator, wherein the indicator is associated with the receptor such that in the presence of the analyte the indicator is displaced from the receptor to produce the signal.
282. The system of claim 278, wherein the receptor comprises a polynucleotide.
283. The system of claim 278, wherein the receptor comprises a peptide.
284. The system of claim 278, wherein the receptor comprises an enzyme.
285. The system of claim 278, wherein the receptor comprises a synthetic receptor.
286. The system of claim 278, wherein the receptor comprises an unnatural biopolymer.
287. The system of claim 278, wherein the receptor comprises an antibody.
288. The system of claim 278, wherein the receptor comprises an antigen.
289. The system of claim 255, wherein the analyte comprises bacteria, and wherein the particle is configured to produce the signal in the presence of the bacteria.
290. The system of claim 255, wherein the system comprises a plurality of particles positioned within a plurality of cavities, and wherein the plurality of particles produce a detectable pattern in the presence of the analyte.

291. A sensor array for detecting an analyte in a fluid comprising:  
a supporting member; wherein a first cavity and a second cavity are formed within the supporting member;  
a first particle positioned within the first cavity;  
a second particle positioned within the second cavity, wherein the second particle comprises a reagent,  
wherein a portion of the reagent is removable from the second particle when contacted with a  
decoupling solution, and wherein the reagent is configured to modify the first particle, when the  
reagent is contacted with the first particle, such that the first particle will produce a signal when the  
first particle interacts with the analyte during use;  
a first pump coupled to the supporting member, wherein the pump is configured to direct the fluid  
towards the first cavity;  
a second pump coupled to the supporting member, wherein the second pump is configured to direct the  
decoupling solution towards the second cavity;  
wherein a first channel is formed in the supporting member, the first channel coupling the first pump to  
the first cavity such that the fluid flows through the first channel to the first cavity during use, and  
wherein a second channel is formed in the supporting member, the second channel coupling the second  
cavity to the first cavity such that the decoupling solution flows from the second cavity through the  
second channel to the first cavity during use.
292. The sensor array of claim 291, wherein the first particle comprises a receptor molecule coupled to a  
first polymeric resin, and wherein the second particle comprises an indicator molecule coupled to a  
second polymeric resin.
293. The sensor array of claim 291, wherein the first particle comprises an indicator molecule coupled to a  
first polymeric resin, and the second particle comprises a receptor molecule coupled to a second  
polymeric resin.
294. The sensor array of claim 291, wherein the first particle comprises a first polymeric resin configured to  
bind to the receptor molecule, and wherein the second particle comprises the receptor molecule  
coupled to a second polymeric resin.
295. The sensor array of claim 291, wherein the supporting member comprises a plastic material.
296. The sensor array of claim 291, wherein the supporting member comprises a silicon wafer.
297. The sensor array of claim 291, wherein the supporting member comprises a dry film photoresist  
material.
298. The sensor array of claim 291, wherein the supporting member comprises a plurality of layers of a dry

film photoresist material.

299. The sensor array of claim 291, wherein an inner surface of the first cavity is coated with a reflective material.
- 5 300. The sensor array of claim 291, further comprising a detector coupled to the bottom surface of the supporting member, wherein the detector is positioned below the first cavity.
- 10 301. The sensor array of claim 291, further comprising a plurality of additional particles positioned within a plurality of additional cavities formed in the supporting member, and wherein the second cavity is coupled to the additional cavities such that the reagent may be transferred from the second particle to the additional cavities during use.
- 15 302. The sensor array of claim 291, wherein the first and second pumps comprise a diaphragm pump.
303. The sensor array of claim 291, wherein the first and second pumps comprise an electrode pump.
304. The sensor array of claim 291, wherein the first pump comprises a diaphragm pump or an electrode pump and wherein the second pump comprises a diaphragm pump or an electrode pump.
- 20 305. The sensor array of claim 291, wherein the first cavity is substantially tapered such that the width of the first cavity narrows in a direction from a top surface of the supporting member toward a bottom surface of the supporting member, and wherein a minimum width of the first cavity is substantially less than a width of the first particle, and wherein the second cavity is substantially tapered such that the width of the second cavity narrows in a direction from a top surface of the supporting member toward a bottom surface of the supporting member, and wherein a minimum width of the second cavity is substantially less than a width of the second particle.
- 25 306. The sensor array of claim 291, wherein a width of a bottom portion of the first cavity is substantially less than a width of a top portion of the first cavity, and wherein the width of the bottom portion of the first cavity is substantially less than a width of the first particle, and wherein a width of a bottom portion of the second cavity is substantially less than a width of a top portion of the second cavity, and wherein the width of the bottom portion of the second cavity is substantially less than a width of the second particle.
- 30 307. The sensor array of claim 291, further comprising a reservoir coupled to the second pump, the reservoir configured to hold the decoupling solution.
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308. A system for detecting an analyte in a fluid comprising:

a light source;

a sensor array, the sensor array comprising:

a supporting member; wherein a first cavity and a second cavity are formed within the supporting

5 member;

a first particle positioned within the first cavity;

a second particle positioned within the second cavity, wherein the second particle comprises a reagent, wherein a portion of the reagent is removable from the second particle when

10 contacted with a decoupling solution, and wherein the reagent is configured to modify the first particle, when the reagent is contacted with the first particle, such that the first particle will

produce a signal when the first particle interacts with the analyte during use;

a first pump coupled to the supporting member, wherein the pump is configured to direct the fluid towards the first cavity;

15 a second pump coupled to the supporting member, wherein the second pump is configured to direct the decoupling solution towards the second cavity;

wherein a first channel is formed in the supporting member, the first channel coupling the first pump to the first cavity such that the fluid flows through the first channel to the first cavity

during use, and wherein a second channel is formed in the supporting member, the second channel coupling the second cavity to the first cavity such that the decoupling solution flows

20 from the second cavity through the second channel to the first cavity during use; and

a detector, the detector being configured to detect the signal produced by the interaction of the analyte with the particle during use;

wherein the light source and detector are positioned such that light passes from the light source, to the particle, and onto the detector during use.

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309. The system of claim 308, wherein the sensor array further comprises a plurality of additional particles positioned within a plurality of additional cavities, and wherein the system is configured to substantially simultaneously detect a plurality of analytes in the fluid, and wherein the second cavity is coupled to the additional cavities such that the reagent may be transferred from the second particle to the additional cavities during use.

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310. The system of claim 308, wherein the light source comprises a light emitting diode.

311. The system of claim 308, wherein the light source comprises a red light emitting diode, a blue light emitting diode, and a green light emitting diode.

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312. The system of claim 308, wherein the light source comprises a white light source.

313. The system of claim 308, wherein the first particle comprises a receptor molecule coupled to a first

polymeric resin, and wherein the second particle comprises an indicator molecule coupled to a second polymeric resin.

- 5 314. The system of claim 308, wherein the first particle comprises an indicator molecule coupled to a first polymeric resin, and the second particle comprises a receptor molecule coupled to a second polymeric resin.
- 10 315. The system of claim 308, wherein the first particle comprises a first polymeric resin configured to bind to the receptor molecule, and wherein the second particle comprises the receptor molecule coupled to a second polymeric resin.
316. The system of claim 308, wherein the supporting member comprises a plastic material.
- 15 317. The system of claim 308, wherein the supporting member comprises a silicon wafer.
318. The system of claim 308, wherein the supporting member comprises a dry film photoresist material.
- 20 319. The system of claim 308, wherein the supporting member comprises a plurality of layers of a dry film photoresist material.
320. The system of claim 308, wherein an inner surface of the first cavity is coated with a reflective material.
- 25 321. The system of claim 308, wherein the first and second pumps comprise a diaphragm pump.
322. The system of claim 308, wherein the first and second pumps comprise an electrode pump.
- 30 323. The system of claim 308, wherein the first pump comprises a diaphragm pump or an electrode pump and wherein the second pump comprises a diaphragm pump or an electrode pump.
- 35 324. The system of claim 308, wherein the first cavity is substantially tapered such that the width of the first cavity narrows in a direction from a top surface of the supporting member toward a bottom surface of the supporting member, and wherein a minimum width of the first cavity is substantially less than a width of the first particle, and wherein the second cavity is substantially tapered such that the width of the second cavity narrows in a direction from a top surface of the supporting member toward a bottom surface of the supporting member, and wherein a minimum width of the second cavity is substantially less than a width of the second particle.
325. The system of claim 308, wherein a width of a bottom portion of the first cavity is substantially less



- than a width of a top portion of the first cavity, and wherein the width of the bottom portion of the first cavity is substantially less than a width of the first particle, and wherein a width of a bottom portion of the second cavity is substantially less than a width of a top portion of the second cavity, and wherein the width of the bottom portion of the second cavity is substantially less than a width of the second particle.
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326. The system of claim 308, wherein the sensor array further comprises a reservoir coupled to the second pump, the reservoir configured to hold the decoupling solution.
- 10 327. The system of claim 308, wherein the analyte comprises bacteria, and wherein the first particle is configured to produce the signal in the presence of the bacteria.
328. A method for forming a sensor array configured to detect an analyte in a fluid, comprising:  
forming a cavity in a supporting member, wherein the cavity is configured to allow the fluid to pass  
15 through the supporting member;  
placing a particle in the cavity, wherein the particle is configured to produce a signal when the particle interacts with the analyte; and  
placing a cover upon a portion of the supporting member, wherein the cover is configured to inhibit dislodgment of the particle from the cavity.
- 20 329. The method of claim 328, further comprising forming a substantially transparent layer upon a bottom surface of the supporting member below the cavity, wherein the bottom layer is configured to inhibit the displacement of the particle from the cavity while allowing the fluid to pass through the supporting member.
- 25 330. The method of claim 328, further comprising forming an optical detector upon a bottom surface of the supporting member below the cavity.
- 30 331. The system of claim 328, wherein a width of a bottom portion of the cavity is substantially less than a width of a top portion of the cavity, and wherein the width of the bottom portion of the cavity is substantially less than a width of the particle.
- 35 332. The method of claim 328, further comprising forming channels in the supporting member wherein the channels are configured to allow the fluid to pass through the supporting member to and from the cavity.
333. The method of claim 328, further comprising forming a pump on the supporting member, the pump being configured to pump the fluid to the cavity.

334. The method of claim 328, further comprising forming additional cavities in the supporting member and further comprising placing additional particles in the additional cavities.
335. The method of claim 328, further comprising forming a cover, wherein forming the cover comprises:  
5 forming a removable layer upon the upper surface of the supporting member;  
forming a cover upon the removable layer;  
forming support structures upon the supporting member, the support structures covering a portion of the cover; and  
dissolving the removable layer.
- 10 336. The method of claim 335, wherein the cover layer is formed prior to forming the cavity.
337. The method of claim 335, wherein forming the cover further comprises forming openings in the cover, wherein the openings are substantially aligned with the cavity.
- 15 338. The method of claim 328, wherein the particles are placed in the cavities using a micromanipulator.
339. The method of claim 328, further comprising forming additional cavities within the supporting member, and further comprising placing additional particles in the additional cavities, wherein placing  
20 the additional particles in the additional cavities comprises:  
placing a first masking layer on the supporting member, wherein the first masking layer covers a first portion of the additional cavities such that passage of a particle into the first portion of the additional cavities is inhibited, and wherein the first masking layer a second portion of the cavities substantially  
unmasked,;  
25 placing the additional particles on the supporting member; and  
moving the additional particles across the supporting member such that the particles fall into the second portion of the cavities.
340. The method of claim 339, further comprising:  
30 removing the first masking layer;  
placing a second masking layer upon the supporting member, wherein the second masking layer covers the second portion of the cavities and a portion of the first portion of the cavities while leaving a third portion of the cavities unmasked;  
placing additional particles on the supporting member; and  
35 moving the additional particles across the supporting member such that the particle fall into the third portion of the cavities.
341. The method of claim 328, wherein forming the cover comprises coupling the cover to the supporting member at a distance above the supporting member substantially less than a width of the particle.

342. The method of claim 328, wherein the supporting member comprises a silicon wafer.
343. The method of claim 342, wherein forming the cavity comprises anisotropically etching the silicon wafer.
- 5 344. The method of claim 342, wherein forming the cavity comprises anisotropically etching the silicon wafer such that the width of the cavity narrows in a direction from a top surface of the supporting member toward a bottom surface of the supporting member, and wherein a minimum width of the cavity is substantially less than a width of the particle.
- 10 345. The method of claim 328, wherein the supporting member comprises a dry film photoresist material.
346. The method of claim 328, wherein the supporting member comprises a plurality of layers of a dry film photoresist material.
- 15 347. The method of claim 346, wherein forming the cavity comprises:  
etching a first opening through a first dry film photoresist layer, the first opening having a width substantially less than a width of the particle;  
placing a second dry film photoresist layer upon the first dry film photoresist layer;  
20 etching a second opening through the second dry film photoresist layer, the second opening being substantially aligned with the first opening, wherein a width of the second opening is substantially greater than the width of the first opening.
348. The method of claim 347, wherein the second dry film photoresist layer comprises a thickness  
25 substantially greater than a width of the particle.
349. The method of claim 345, further comprising forming a reflective layer upon the inner surface of the cavity.
- 30 350. The method of claim 328, wherein the supporting material comprises a plastic material.
351. The method of claim 350, wherein the cavity is formed by drilling the supporting material.
352. The method of claim 350, wherein the cavity is formed by transfer molding the supporting member.
- 35 353. The method of claim 350, wherein the cavity is formed by a punching device.
354. A sensor array produced by the method of claim 328.

355. A sensor array produced by the method of claim 342.
356. A sensor array produced by the method of claim 345.
- 5 357. A sensor array produced by the method of claim 346.
358. A sensor array produce by the method of claim 350.
- 10 359. A method of sensing an analyte in a fluid comprising:  
passing a fluid over a sensor array, the sensor array comprising at least one particle positioned within a  
cavity of a supporting member, wherein the cavity is configured such that the fluid entering the cavity  
passes through the supporting member;  
monitoring a spectroscopic change of the particle as the fluid is passed over the sensor array, wherein  
the spectroscopic change is caused by the interaction of the analyte with the particle.
- 15 360. The method of claim 359, wherein the spectroscopic change comprises a change in absorbance of the  
particle.
361. The method of claim 359, wherein the spectroscopic change comprises a change in fluorescence of the  
20 particle.
362. The method of claim 359, wherein the spectroscopic change comprises a change in phosphorescence of  
the particle.
- 25 363. The method of claim 359, wherein the analyte is a proton atom, and wherein the spectroscopic change  
is produced when the pH of the fluid is varied, and wherein monitoring the spectroscopic change of the  
particle allows the pH of the fluid to be determined.
364. The method of claim 359, wherein the analyte is a metal cation, and wherein the spectroscopic change  
30 is produced in response to the presence of the metal cation in the fluid.
365. The method of claim 359, wherein the analyte is an anion, and wherein the spectroscopic change is  
produced in response to the presence of the anion in the fluid.
- 35 366. The method of claim 359, wherein the analyte is a DNA molecule, and wherein the spectroscopic  
change is produced in response to the presence of the DNA molecule in the fluid.
367. The method of claim 359, wherein the analyte is a protein, and wherein the spectroscopic change is  
produced in response to the presence of the protein in the fluid.

368. The method of claim 359, wherein the analyte is a metabolite, and wherein the spectroscopic change is produced in response to the presence of the metabolite in the fluid.
- 5 369. The method of claim 359, wherein the analyte is a sugar, and wherein the spectroscopic change is produced in response to the presence of the sugar in the fluid.
370. The method of claim 359, wherein the analyte is a bacteria, and wherein the spectroscopic change is produced in response to the presence of the bacteria in the fluid.
- 10 371. The method of claim 359, wherein the particle comprises a receptor coupled to a polymeric resin, and further comprising exposing the particle to an indicator prior to passing the fluid over the sensor array.
372. The method of claim 371, wherein a binding strength of the indicator to the receptor is less than a binding strength of the analyte to the receptor.
- 15 373. The method of claim 371, wherein the indicator is a fluorescent indicator.
374. The method of claim 359, further comprising treating the fluid with an indicator prior to passing the fluid over the sensor array, wherein the indicator is configured to couple with the analyte.
- 20 375. The method of claim 359, wherein the analyte is bacteria and further comprising breaking down the bacteria prior to passing the fluid over the sensor array.
376. The method of claim 359, wherein monitoring the spectroscopic change is performed with a CCD device.
- 25 377. The method of claim 359, further comprising measuring the intensity of the spectroscopic change, and further comprising calculating the concentration of the analyte based on the intensity of the spectroscopic change.
- 30 378. The method of claim 359, wherein monitoring the spectroscopic change comprises:  
directing a red light source at the particle;  
detecting the absorbance of red light by the particle;  
directing a green light source at the particle;  
35 detecting the absorbance of green light by the particle;  
directing a blue light source at the particle; and  
detecting the absorbance of blue light by the particle.

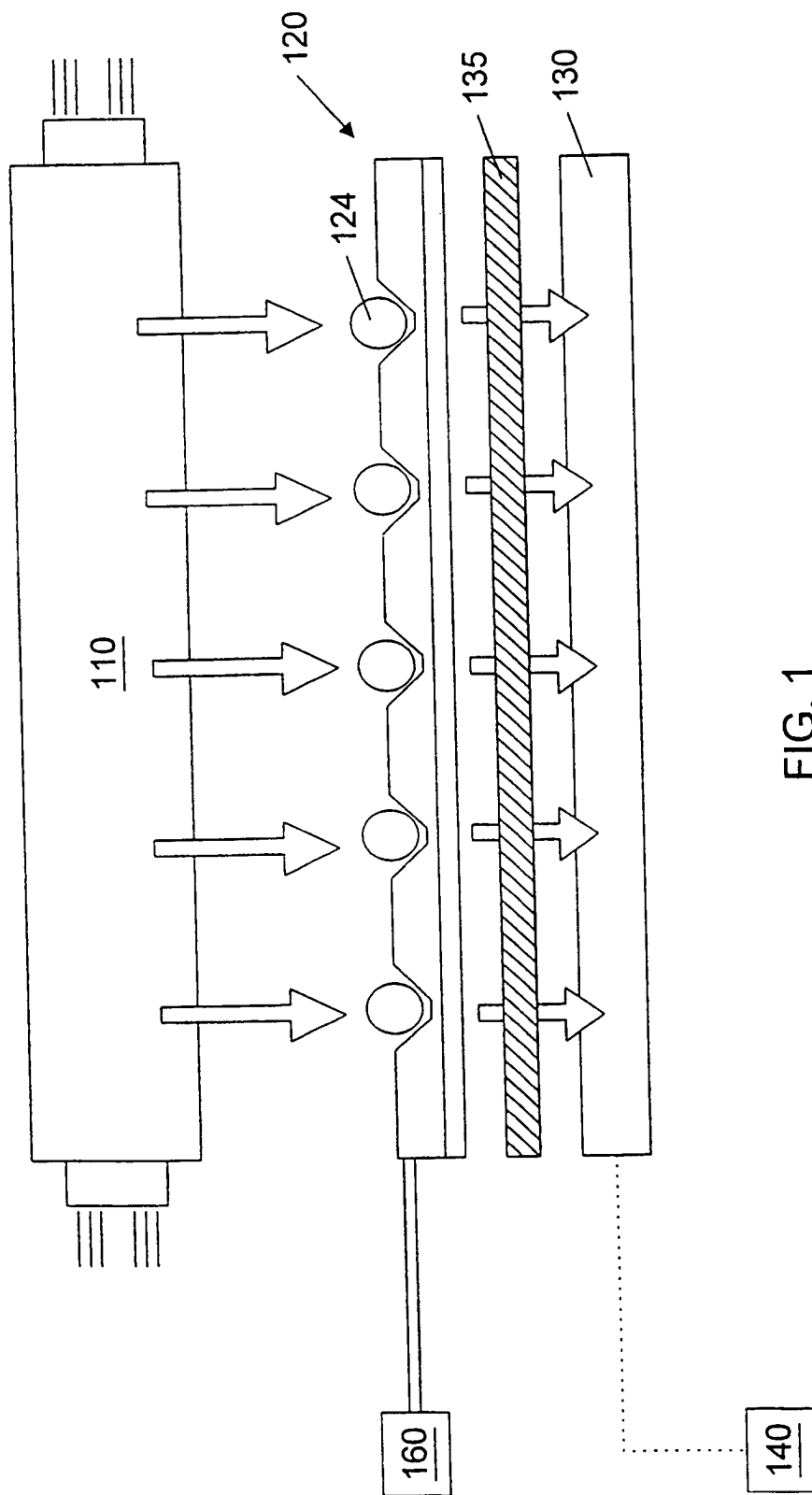
379. A sensor array for detecting an analyte in a fluid comprising:  
at least one particle coupled to a supporting member, wherein the particle is configured to produce  
a signal when the particle interacts with the analyte.
- 5 380. The sensor array of claim 379, wherein the particle is coupled to the supporting member with via an  
adhesive material.
381. The sensor array of claim 379, wherein the particle are coupled to the supporting member via a gel  
material.
- 10 382. The sensor array of claim 379, wherein the particle is suspended in a gel material, the gel material  
covering a portion of the supporting member, and wherein a portion of the particle extends from the  
upper surface of the gel.
- 15 383. The sensor array of claim 379, further comprising a cover positioned above the particle.
384. The sensor array of claim 379, further comprising a cover coupled to the supporting member,  
positioned above the particle, wherein a force exerted by the cover on the particle inhibits the  
displacement of the particle from the supporting member.
- 20 385. The sensor array of claim 379, wherein the particle comprises a receptor molecule coupled to a  
polymeric resin.
386. The sensor array of claim 379, wherein the supporting member comprises a plastic material.
- 25 387. The sensor array of claim 379, wherein the supporting member comprises a dry film photoresist  
material.
388. The sensor array of claim 379, wherein the supporting member comprises a plurality of layers of a dry  
film photoresist material.
- 30 389. The sensor array of claim 379, wherein the supporting member comprises glass.
390. The sensor array of claim 379, further comprising a detector coupled to the bottom surface of the  
supporting member, wherein the detector is positioned below the particle.
- 35 391. The sensor array of claim 379, further comprising a plurality of particles coupled to the supporting  
member.

392. The sensor array of claim 379, wherein the supporting member is composed of a material substantially transparent to visible light.
393. The sensor array of claim 379, wherein the supporting member is composed of a material substantially transparent to ultraviolet light.
394. A system for detecting an analyte in a fluid comprising:  
a light source;  
a sensor array, the sensor array comprising at least one particle coupled to a supporting member,  
wherein the particle is configured to produce a signal when the particle interacts with the analyte, and  
wherein the supporting member is substantially transparent to a portion of light produced by the light source; and  
a detector configured to detect the signal produced by the interaction of the analyte with the particle;  
wherein the light source and detector are positioned such that light passes from the light source, to the particle, and onto the detector during use.
395. The system of claim 394, wherein the system comprises a plurality of additional particles coupled to the supporting member, and wherein the system is configured to substantially simultaneously detect a plurality of analytes in the fluid.
396. The system of claim 394, wherein the light source comprises a light emitting diode.
397. The system of claim 394, wherein the light source comprises a red light emitting diode, a blue light emitting diode, and a green light emitting diode.
398. The system of claim 394, wherein the light source comprises a white light source.
399. The system of claim 394, wherein the particle is coupled to the supporting member with via an adhesive material.
400. The system of claim 394, wherein the particle are coupled to the supporting member via a gel material.
401. The system of claim 394, wherein the particle is suspended in a gel material, the gel material covering a portion of the supporting member, and wherein a portion of the particle extends from the upper surface of the gel.
402. The system of claim 394, wherein the sensor array further comprises a cover positioned above the particle.

403. The system of claim 394, wherein the sensor array further comprises a cover coupled to the supporting member, positioned above the particle, wherein a force exerted by the cover on the particle inhibits the displacement of the particle from the supporting member.
- 5 404. The system of claim 394, wherein the particle comprises a receptor molecule coupled to a polymeric resin.
405. The system of claim 394, wherein the supporting member comprises a plastic material.
- 10 406. The system of claim 394, wherein the supporting member comprises a dry film photoresist material.
407. The system of claim 394, wherein the supporting member comprises a plurality of layers of a dry film photoresist material.
- 15 408. The system of claim 394, wherein the supporting member comprises glass.
409. The system of claim 394, wherein the supporting member is composed of a material substantially transparent to ultraviolet light.
- 20 410. The system of claim 394, wherein the detector comprises a charge-coupled device.
411. The system of claim 394, wherein the particle comprises a receptor molecule coupled to a polymeric resin.
- 25 412. The system of claim 394, wherein the system comprises a plurality of particles coupled to the supporting member, and wherein the plurality of particles produce a detectable pattern in the presence of the analyte.



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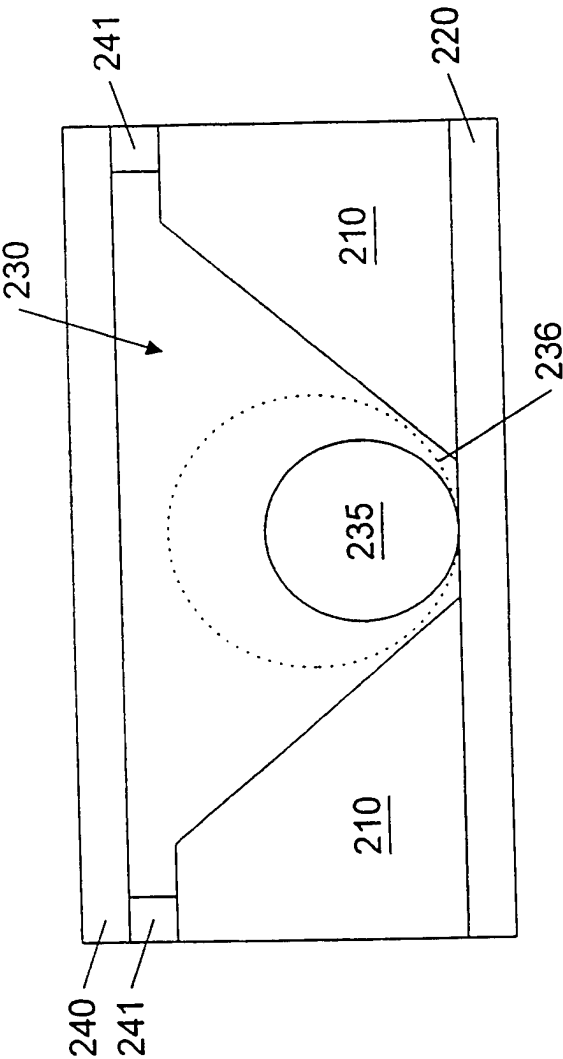


FIG. 2

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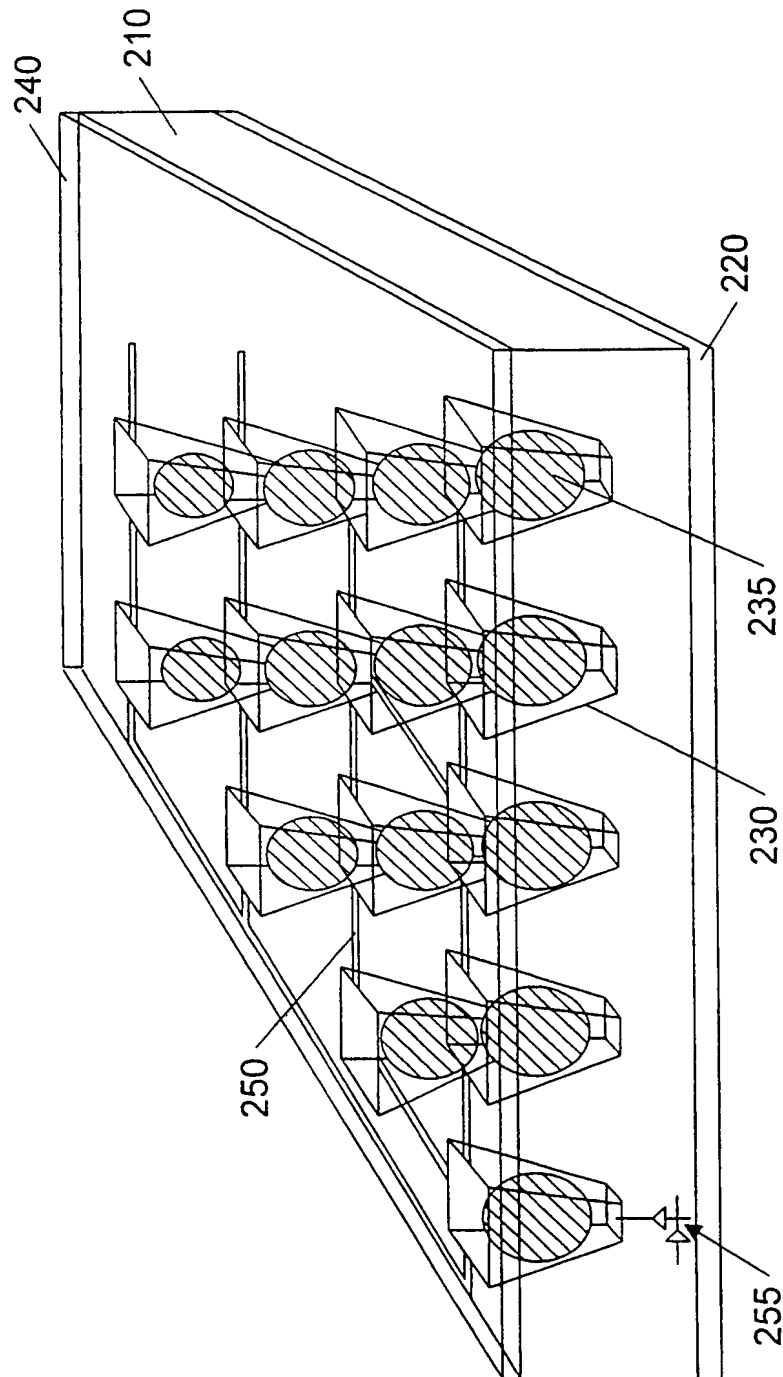


FIG. 3

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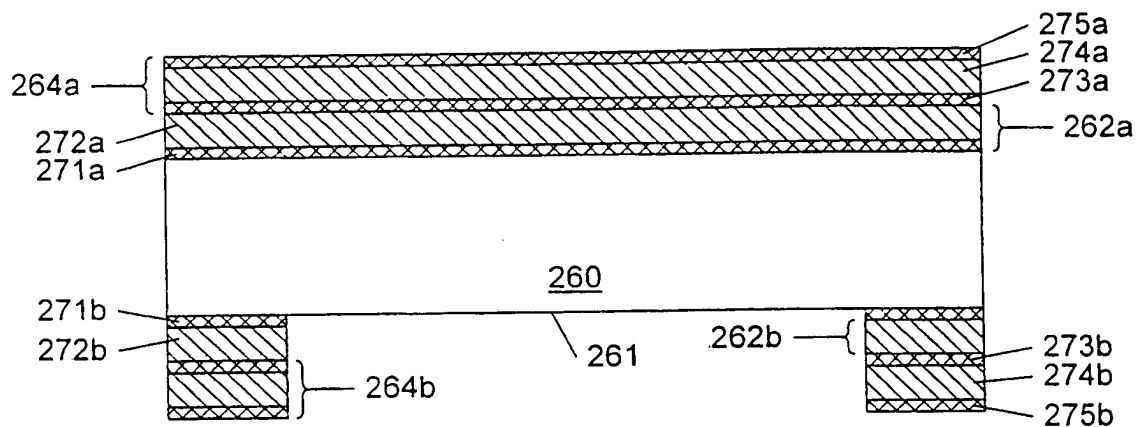


FIG. 4A

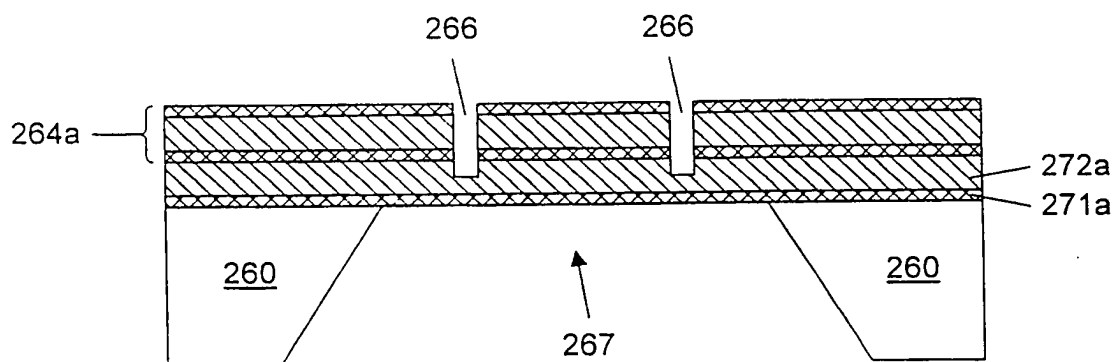


FIG. 4B

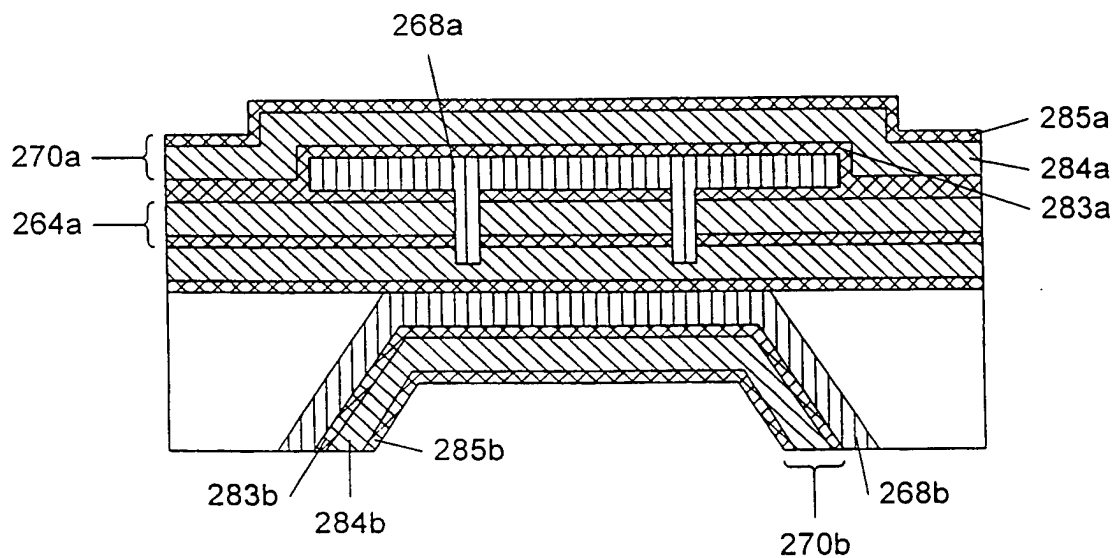


FIG. 4C

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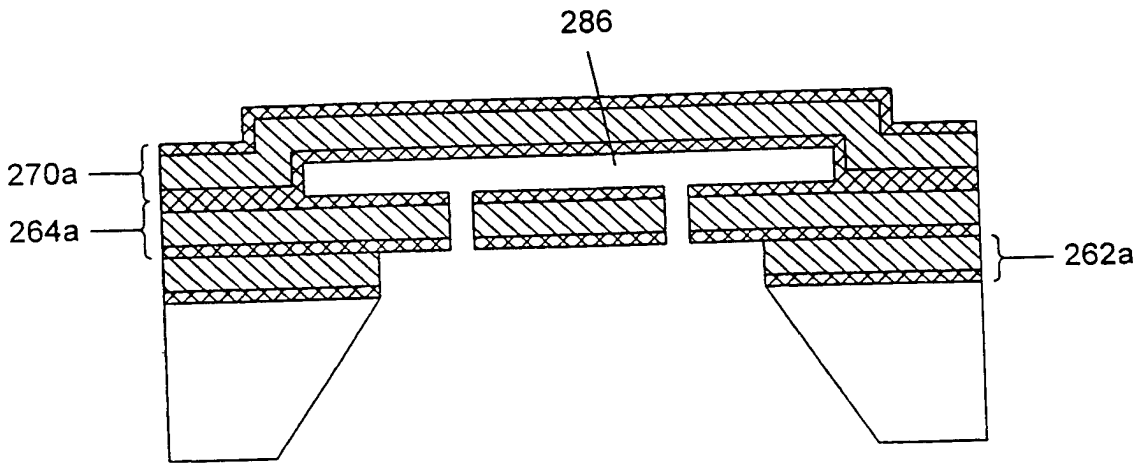


FIG. 4D

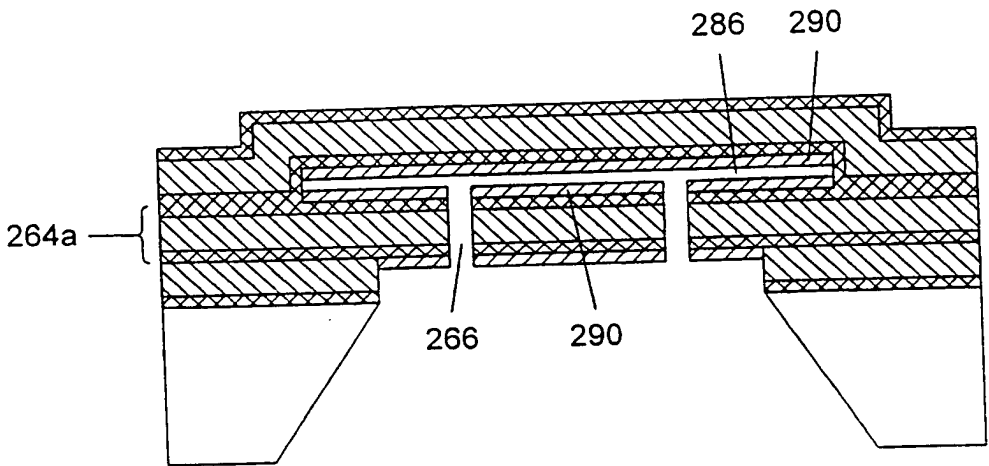


FIG. 4E

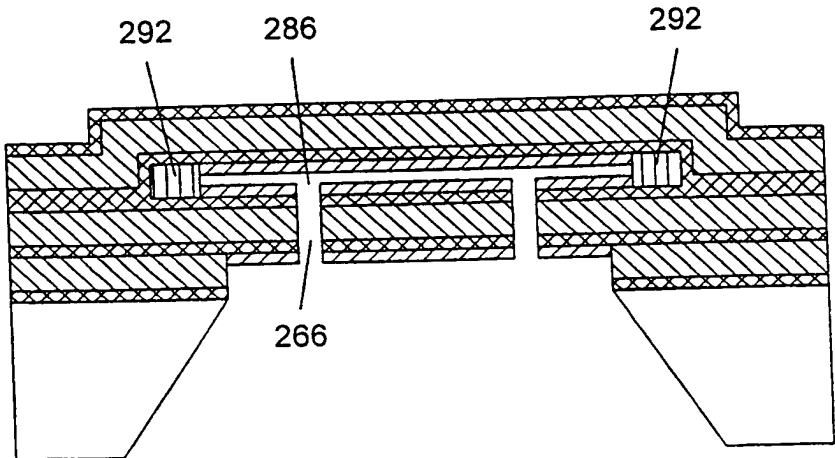


FIG. 4F

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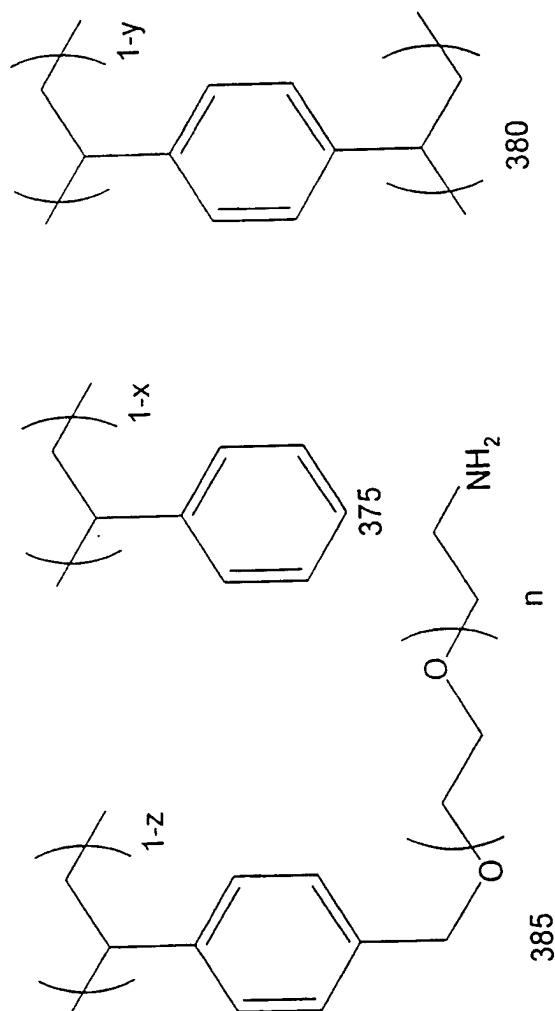


FIG. 5

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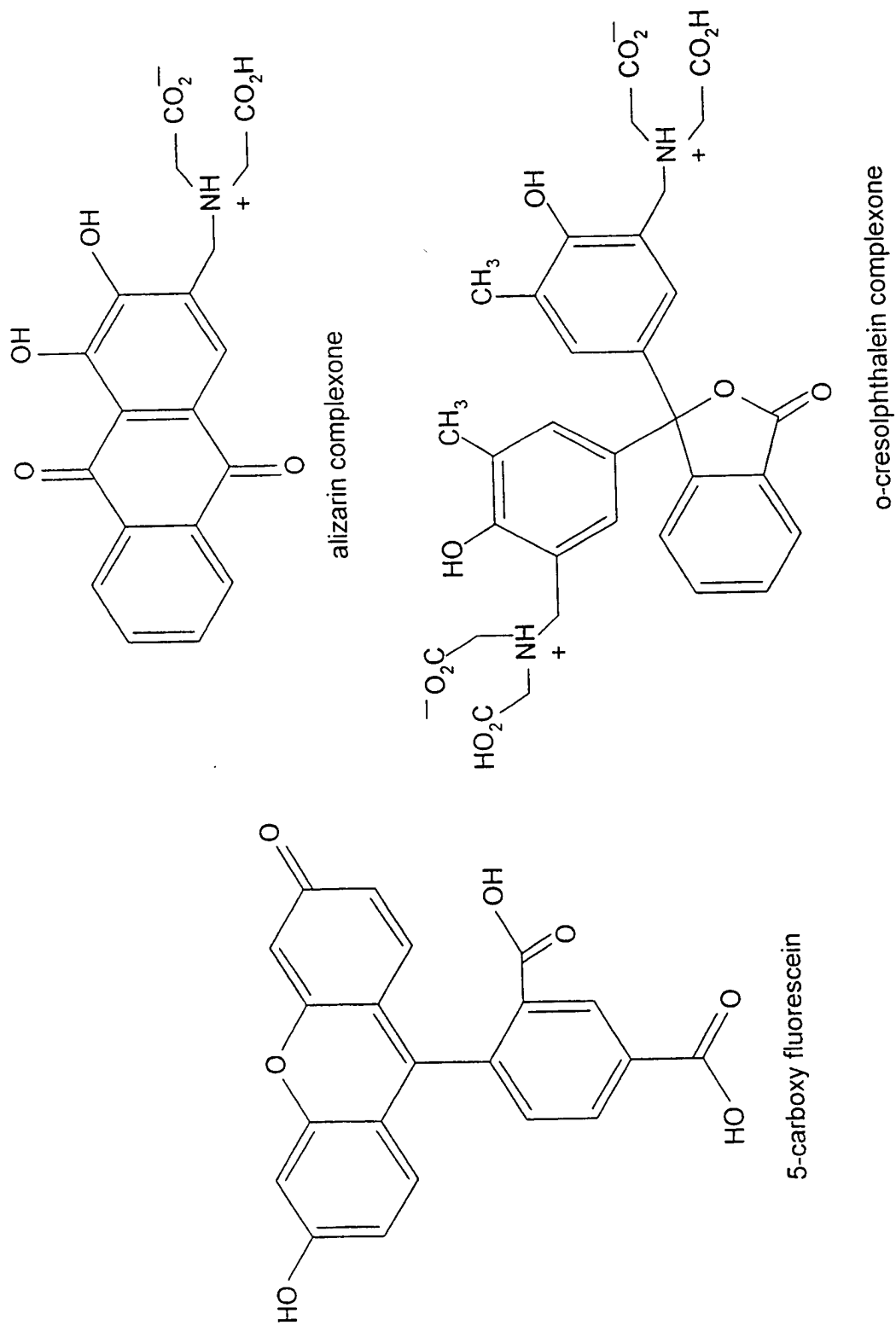


FIG. 6

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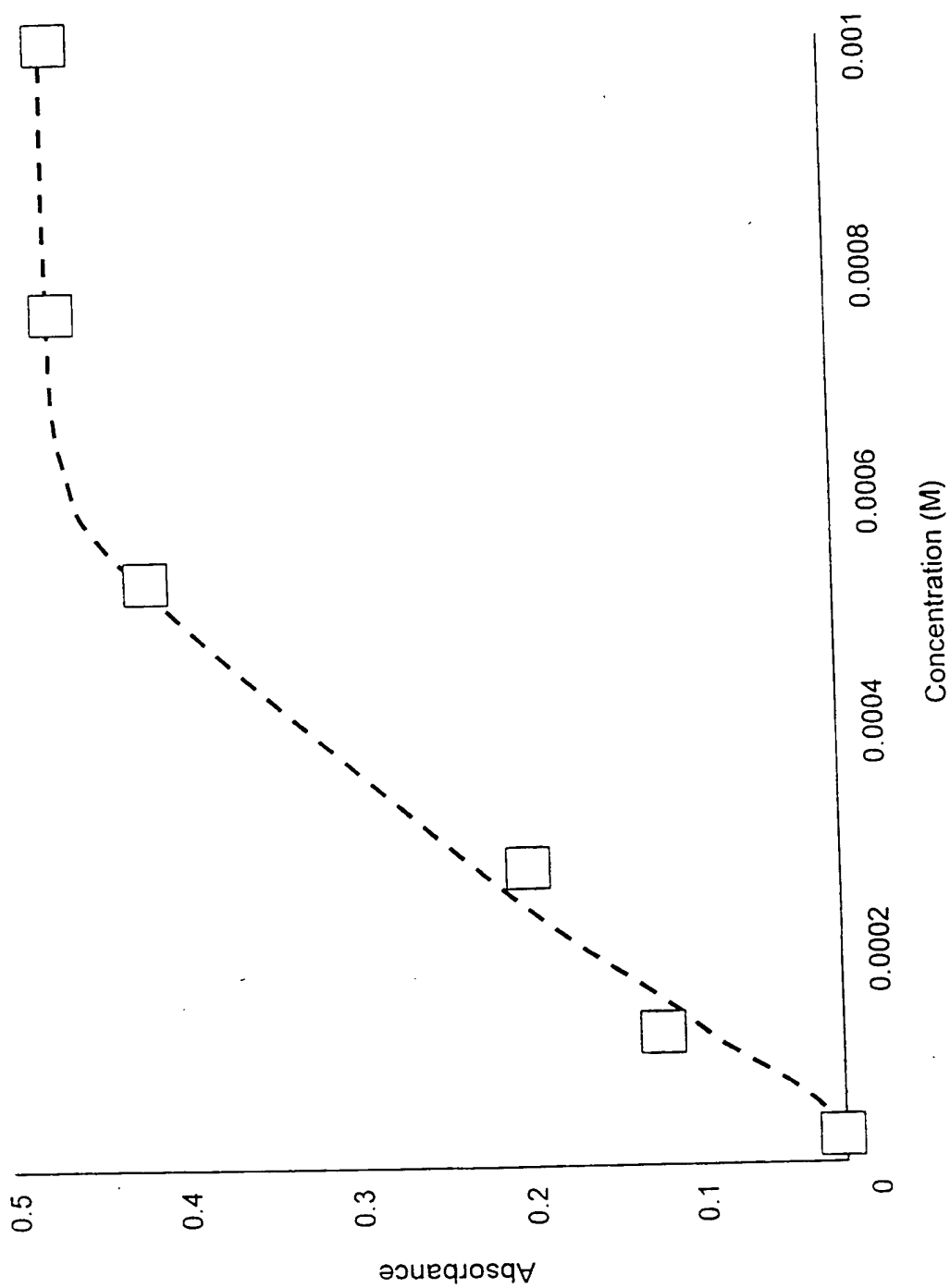


FIG. 7



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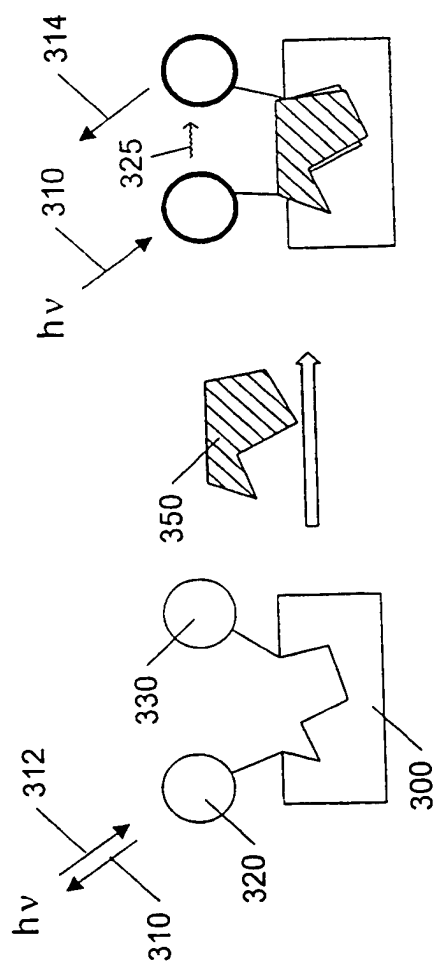


FIG. 8

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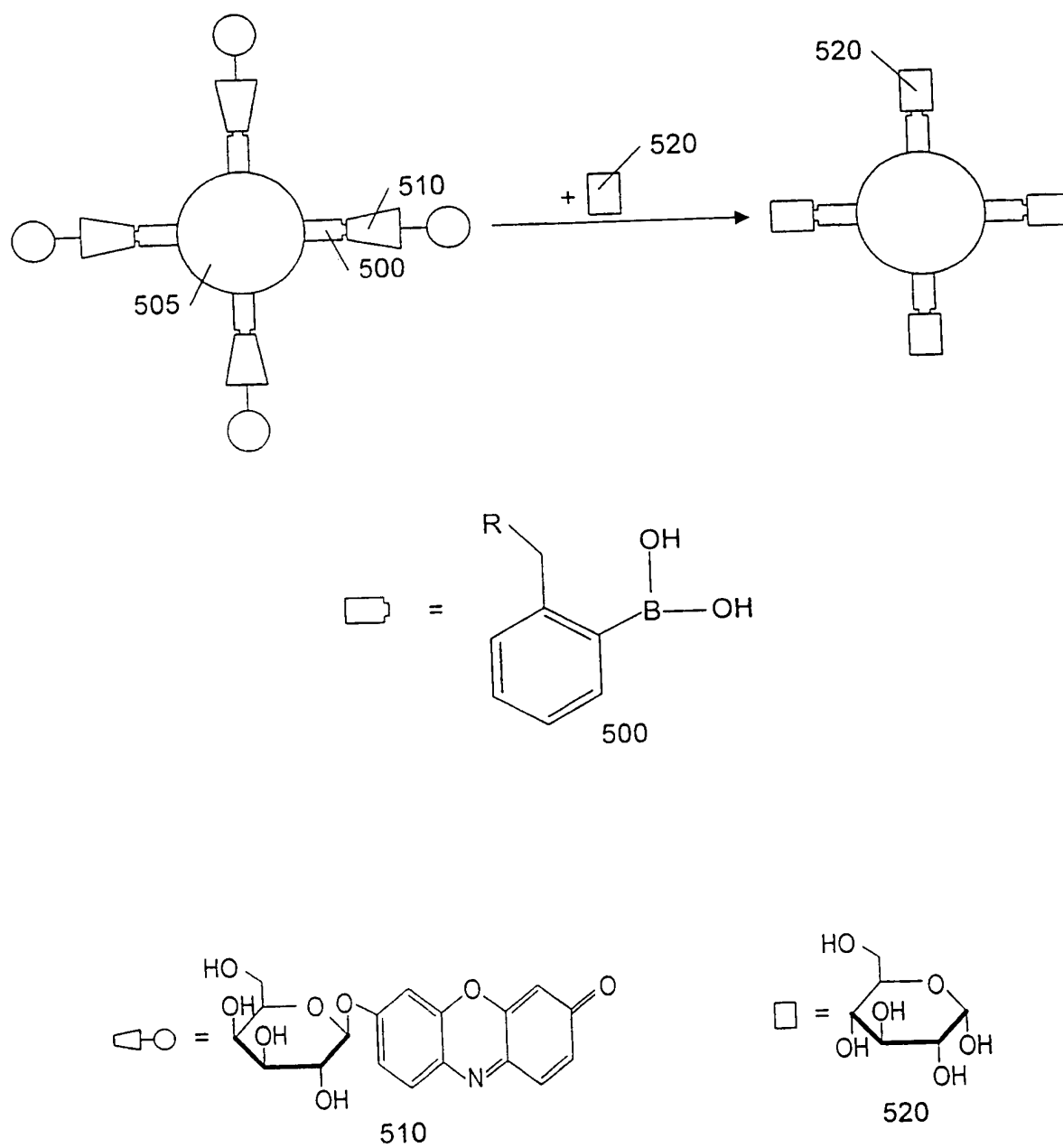
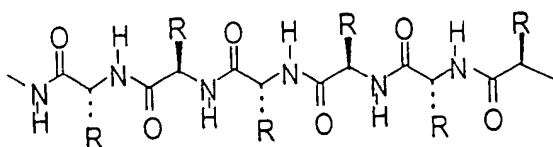
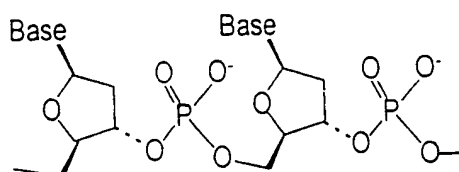


FIG. 9

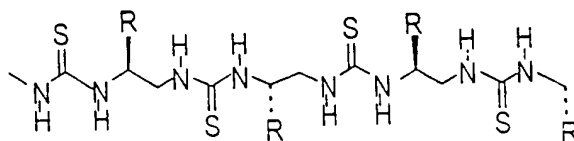
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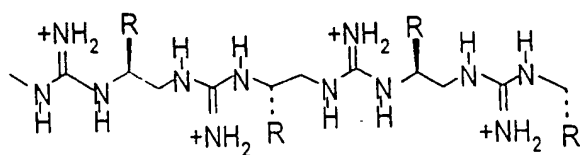
Peptides



Nucleotides



Polythioureas



Polyguanidiniums

FIG. 10

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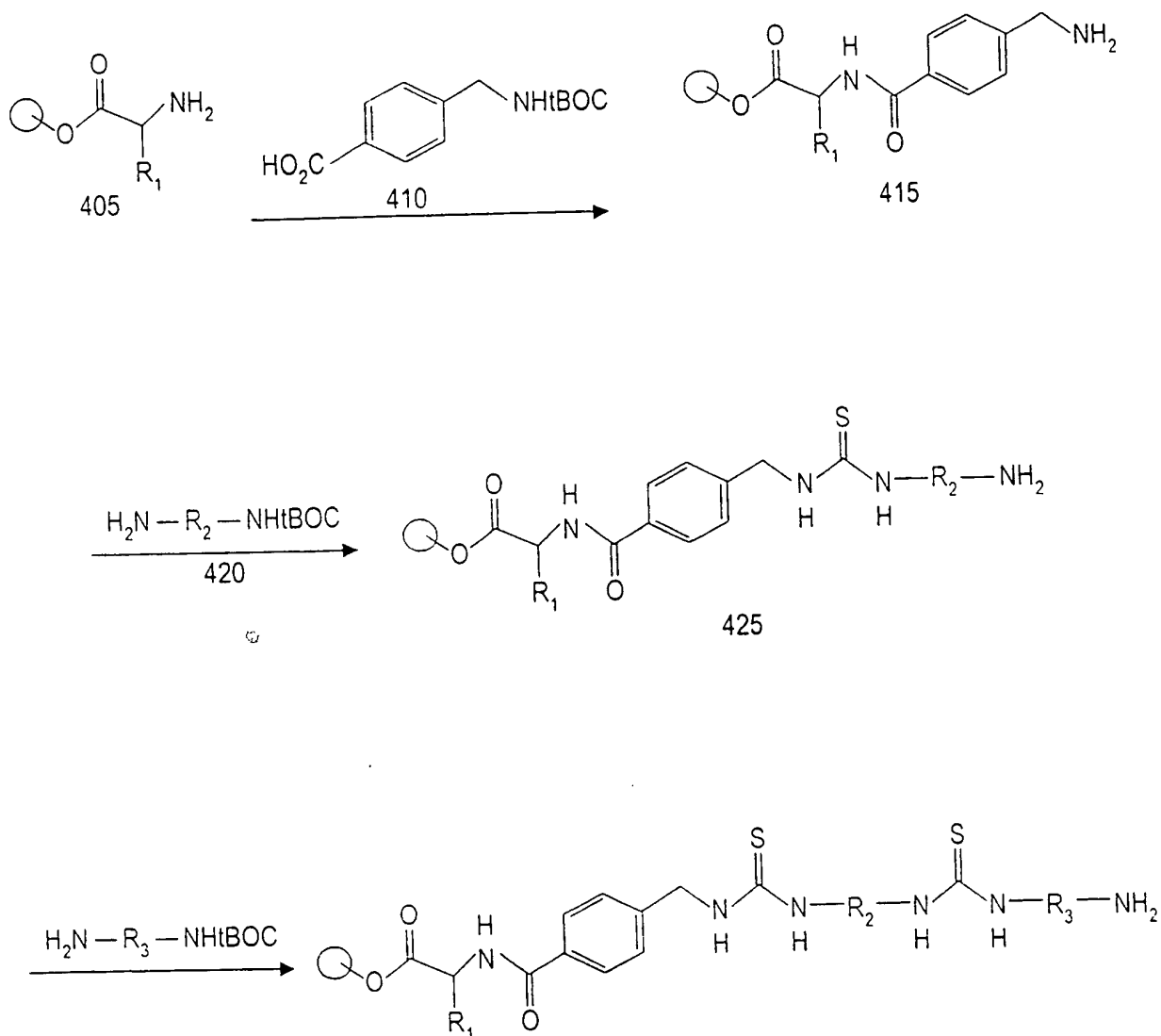


FIG. 11

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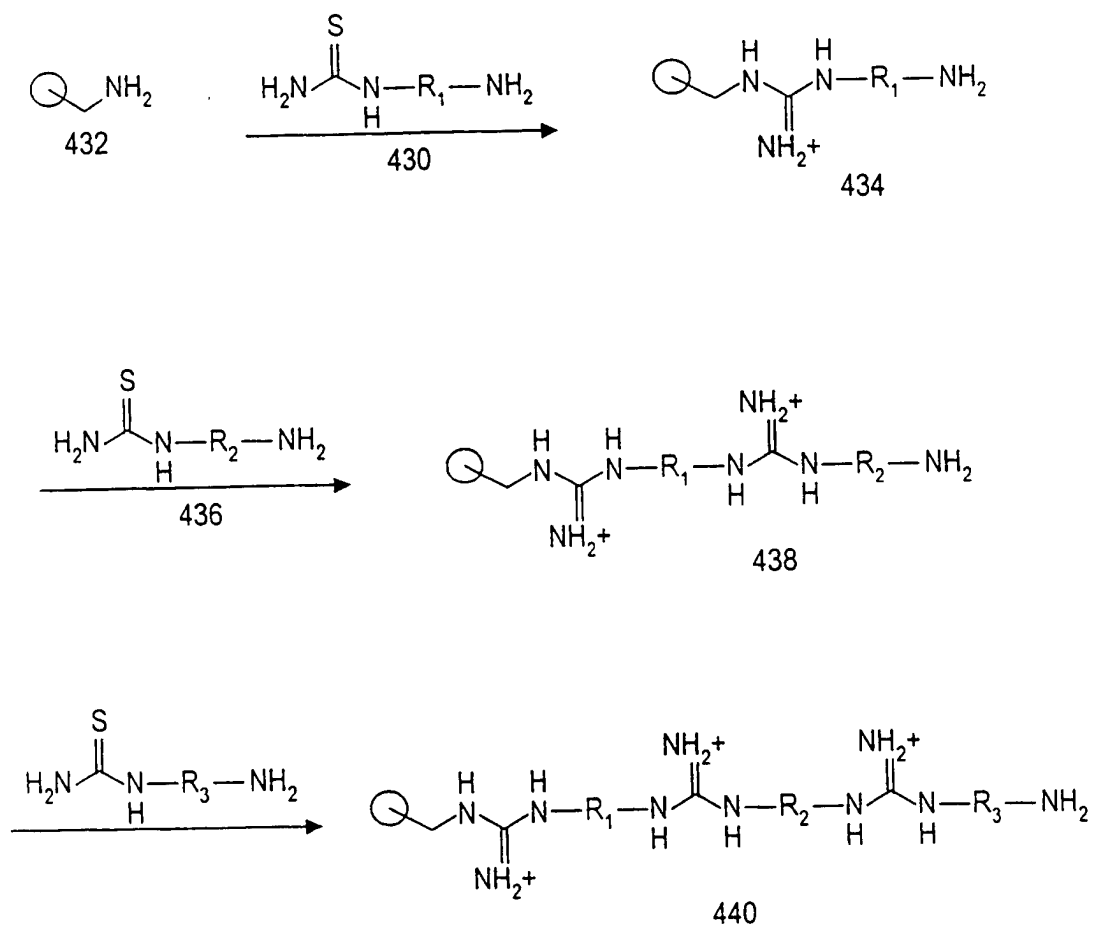


FIG. 12

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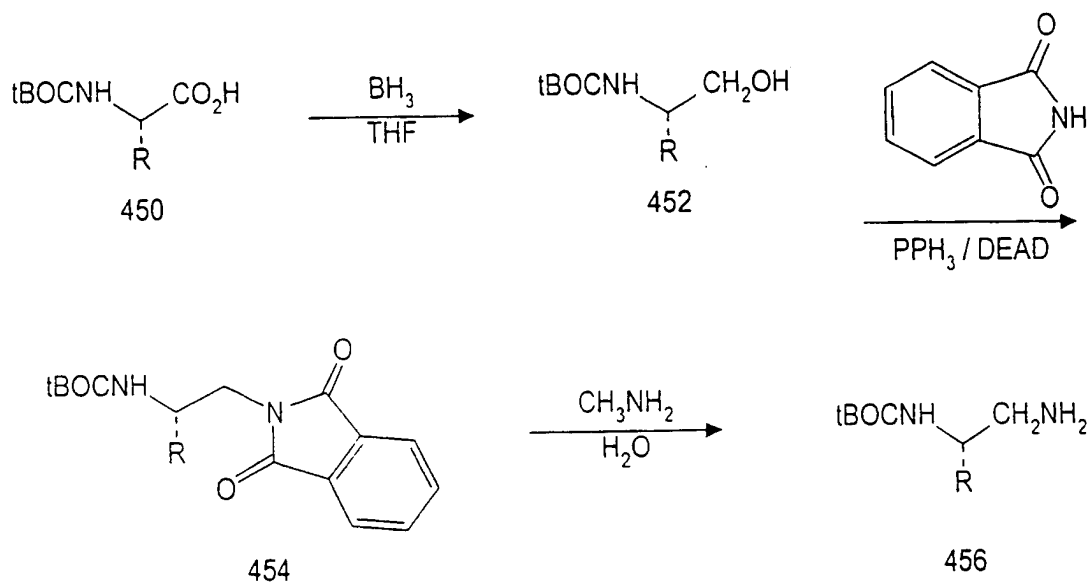


FIG. 13

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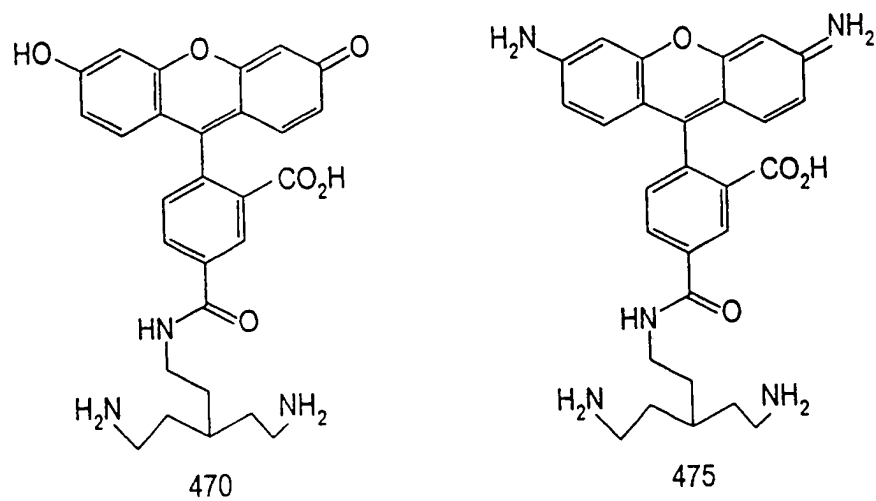


FIG. 14

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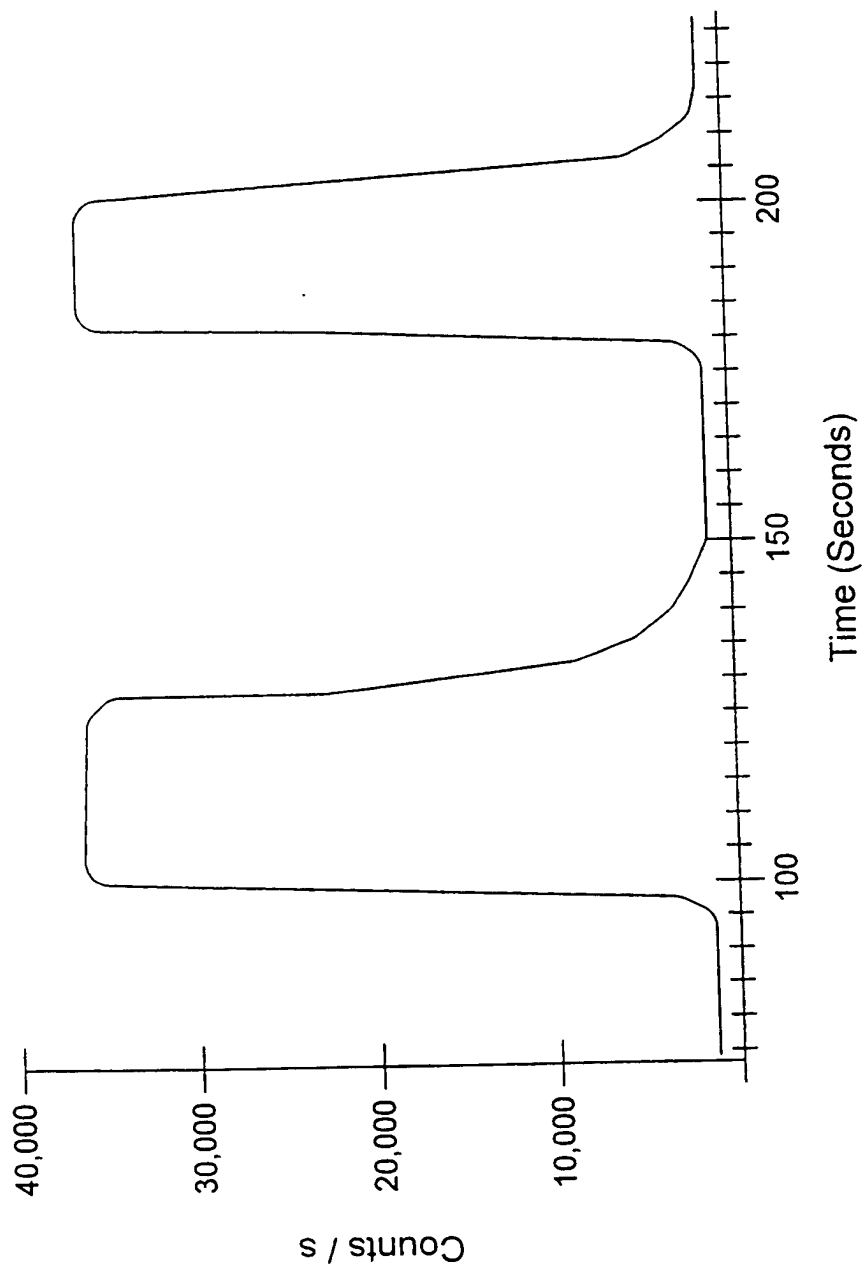


FIG. 15



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RESIN: pH      Ion		Blank	Alizarin	o-Cresol- phthalein	Fluorescein	Alizarin-Ce <sup>3+</sup> complex
2	none					
2	Ca <sup>2+</sup>					
7	none					
7	Ca <sup>2+</sup>					
7	F <sup>-</sup>					
12	none					
12	Ca <sup>2+</sup>					
12	F <sup>-</sup>					

FIG. 16

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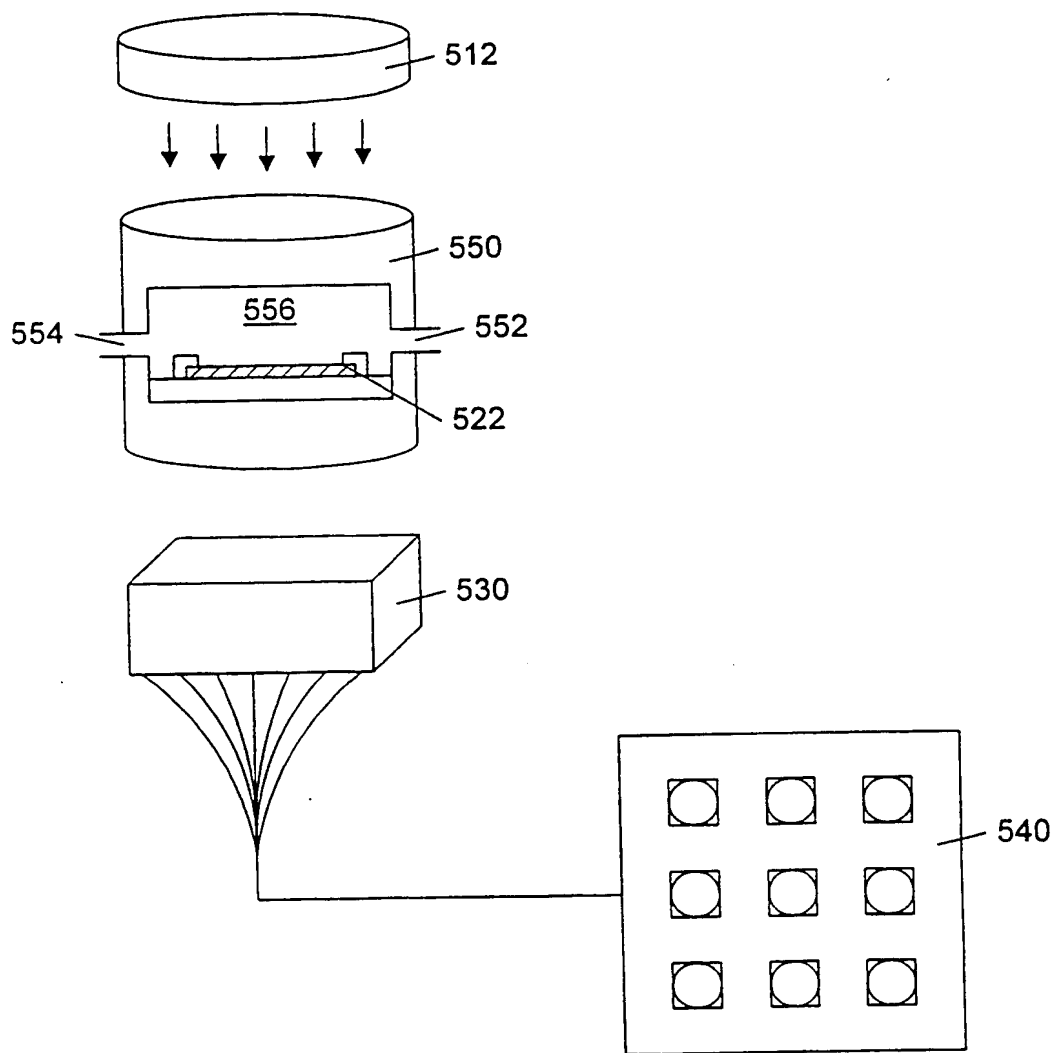


FIG. 17

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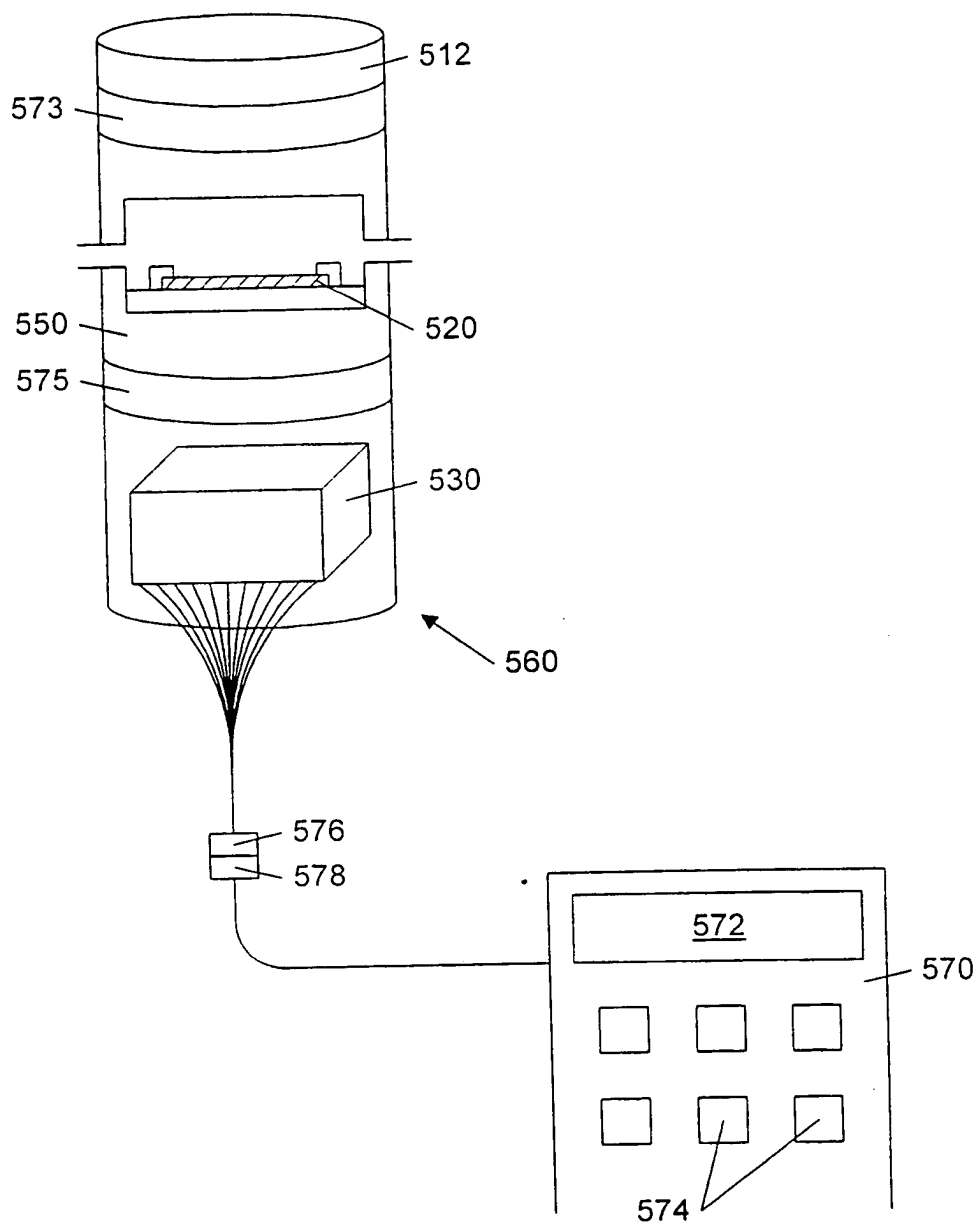


FIG. 18

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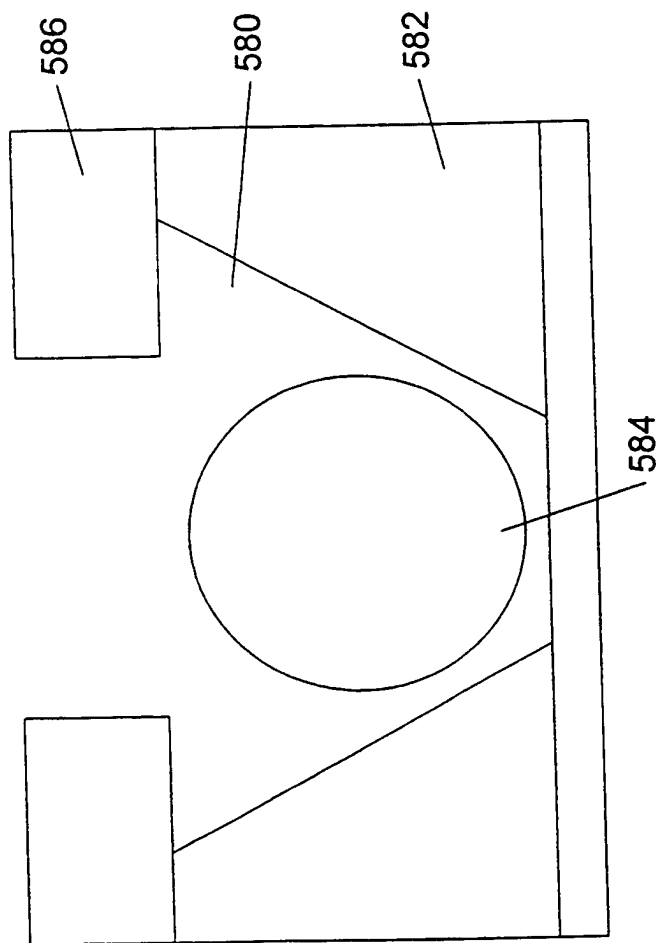


FIG. 19

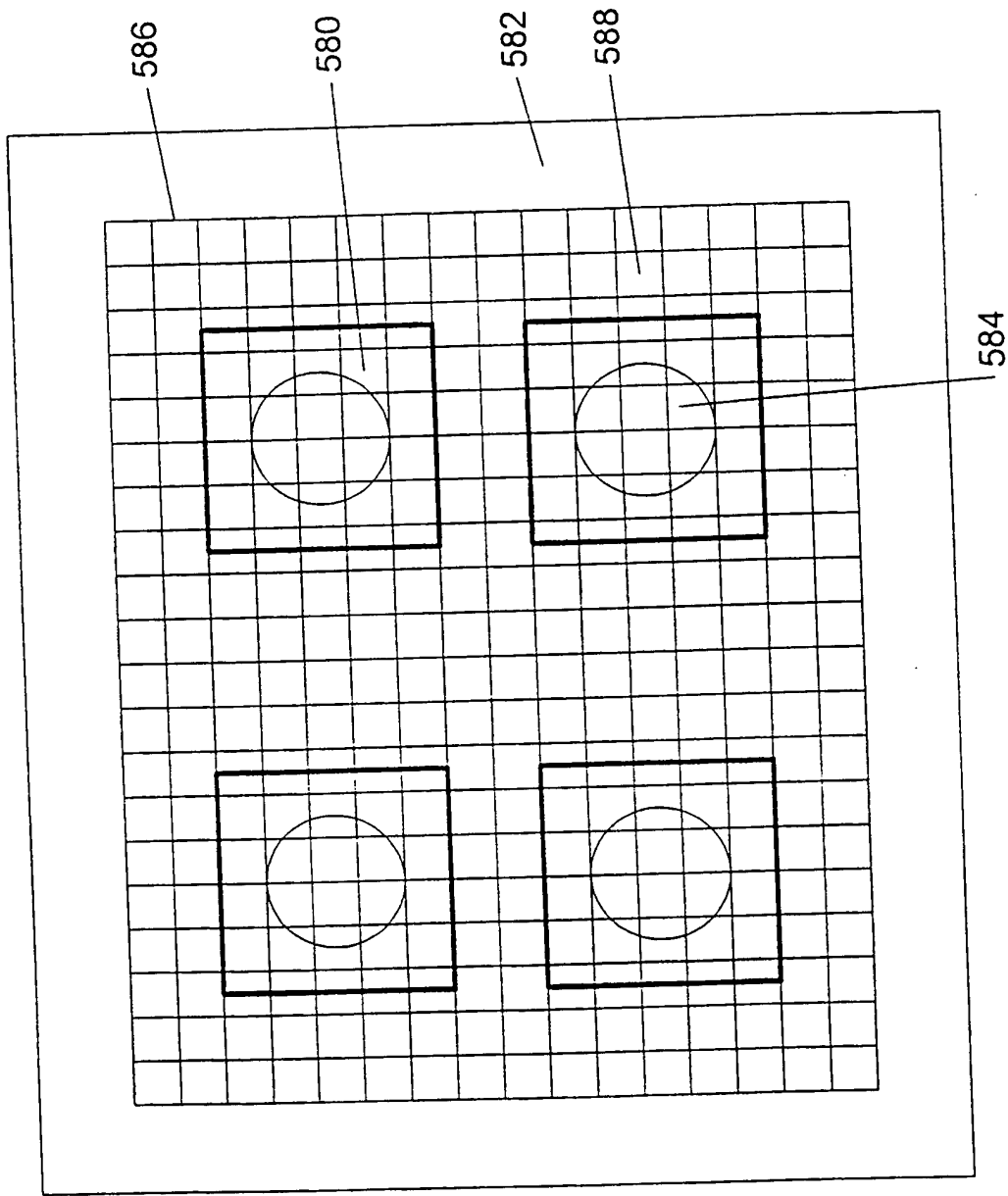


FIG. 20

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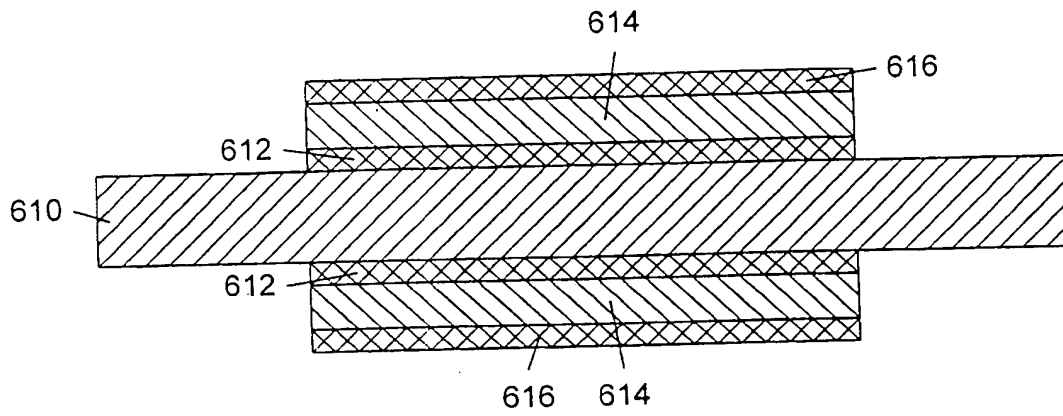


FIG. 21A

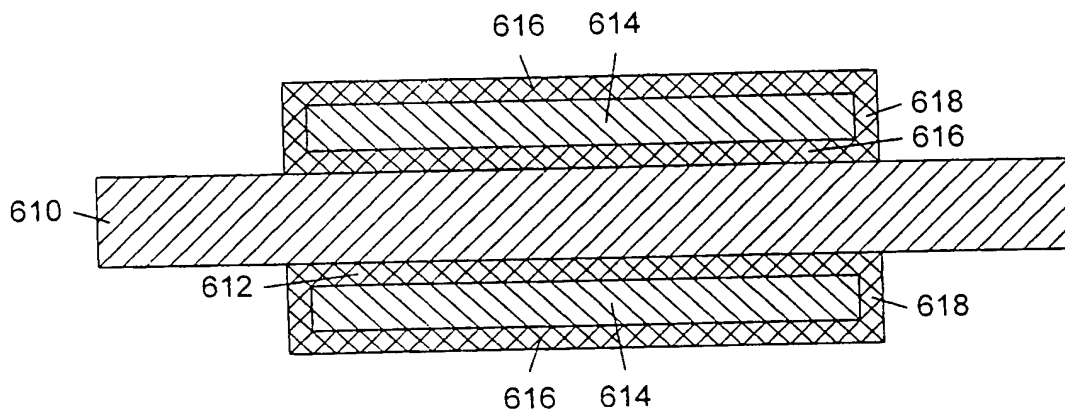


FIG. 21B

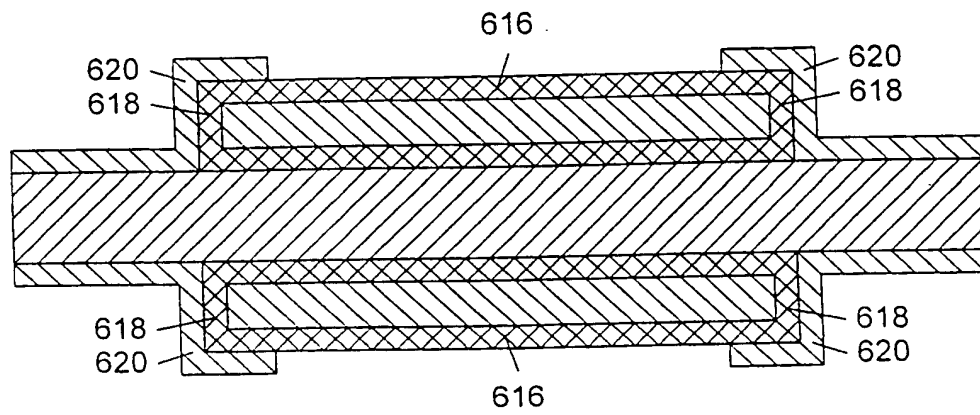


FIG. 21C

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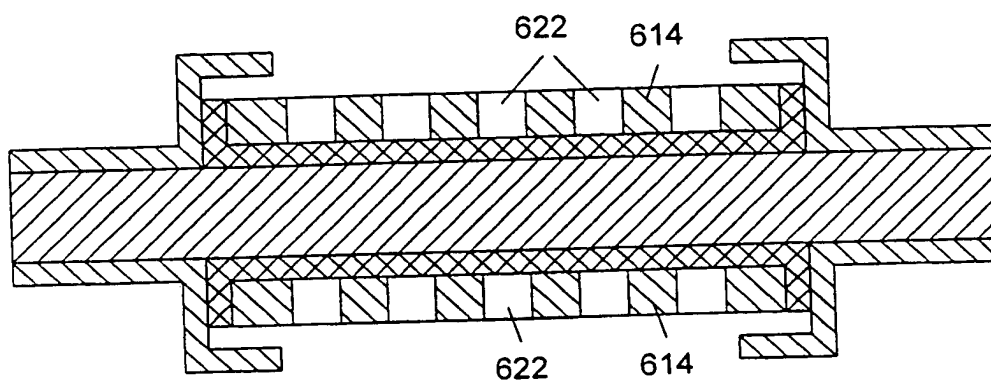


FIG. 21D

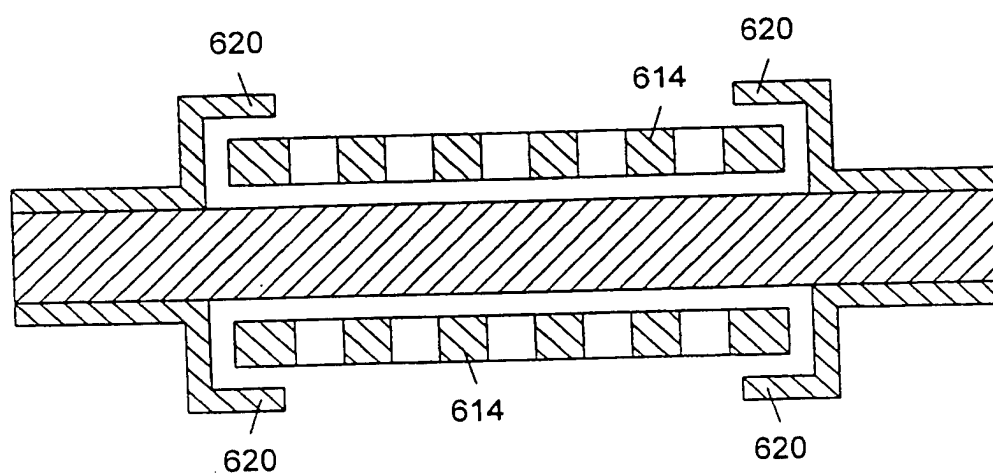


FIG. 21E

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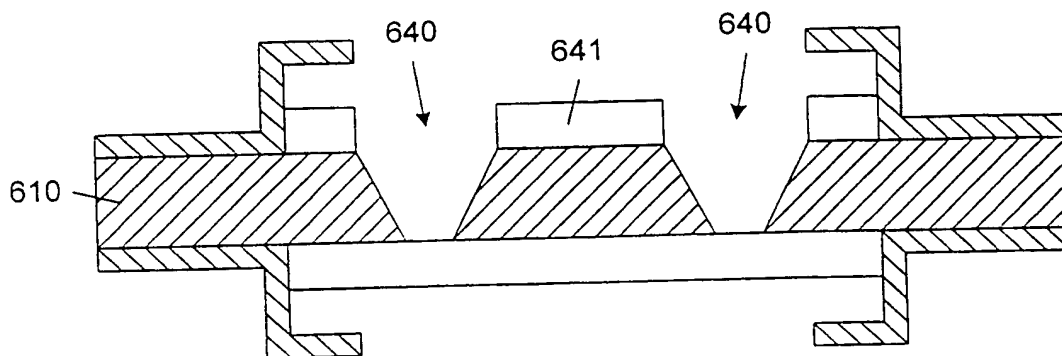


FIG. 21F

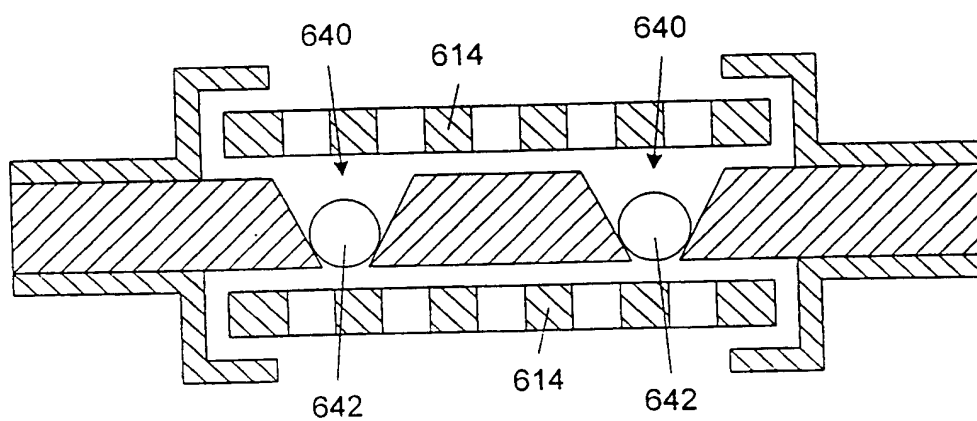


FIG. 21G



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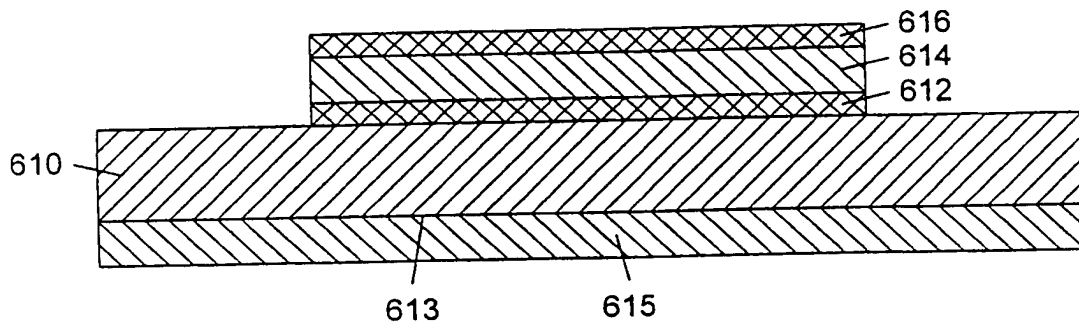


FIG. 22A

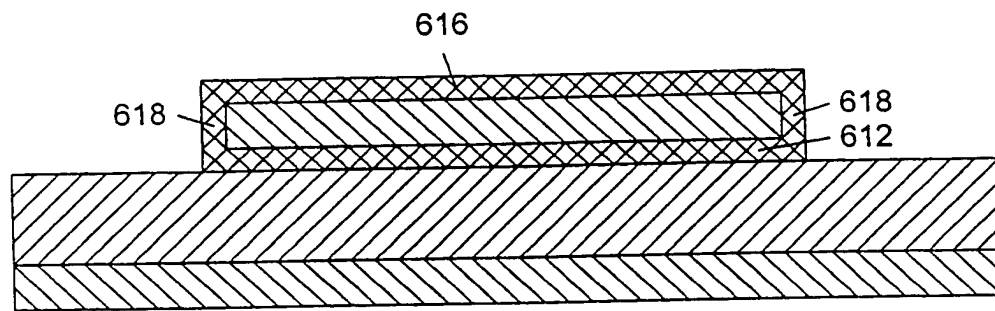


FIG. 22B

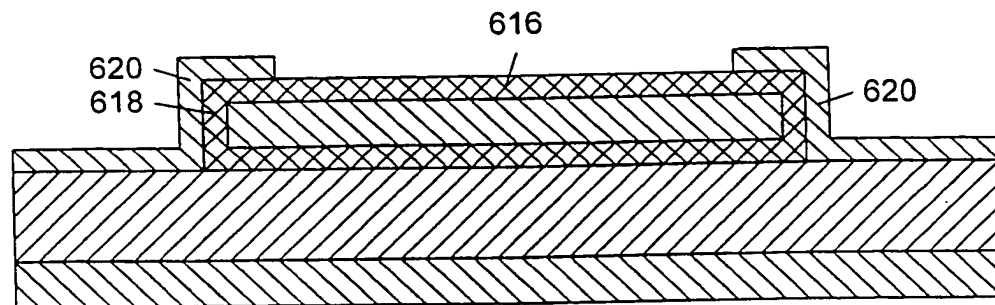


FIG. 22C

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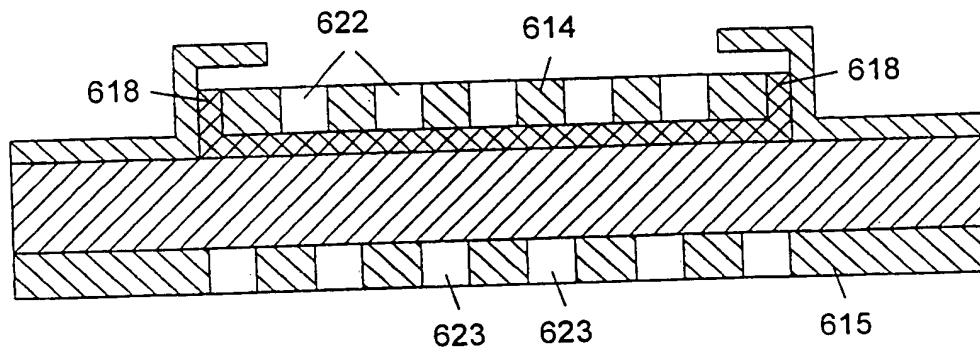


FIG. 22D

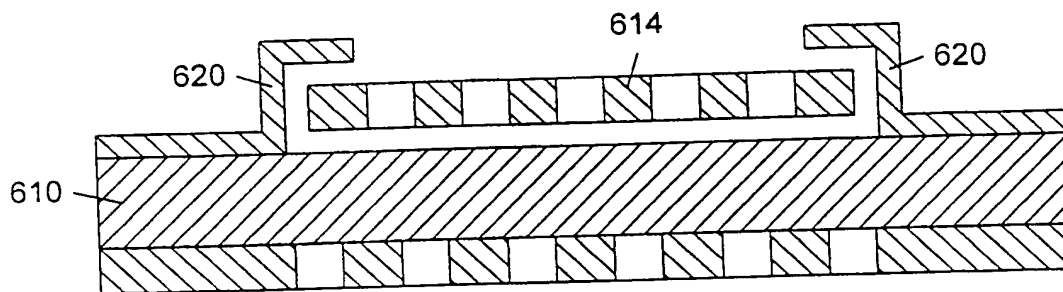


FIG. 22E

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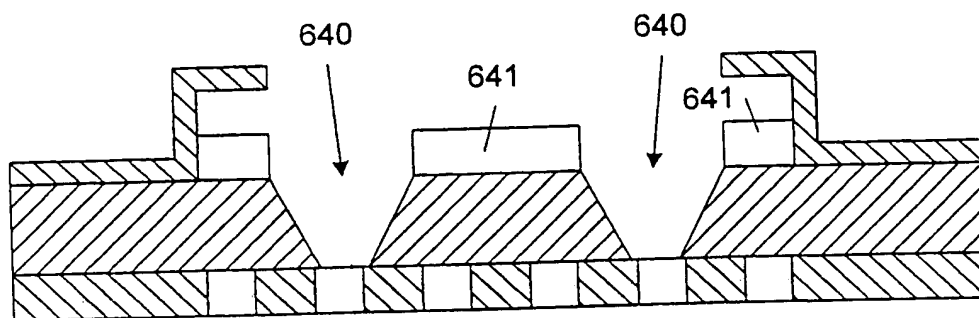


FIG. 22F

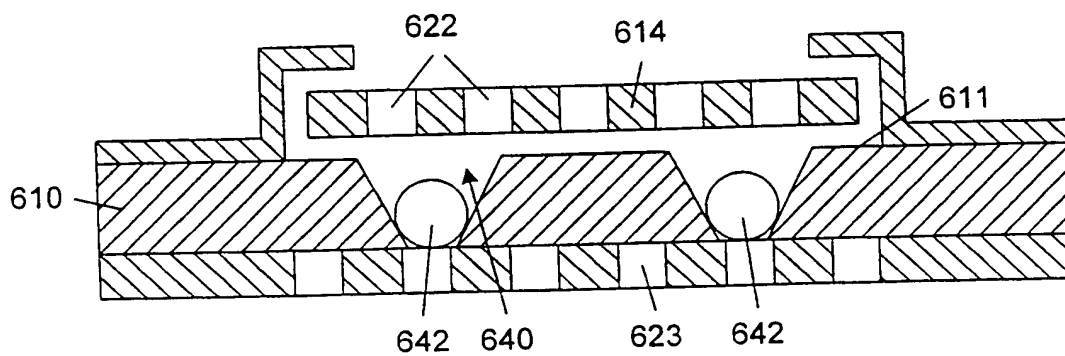


FIG. 22G

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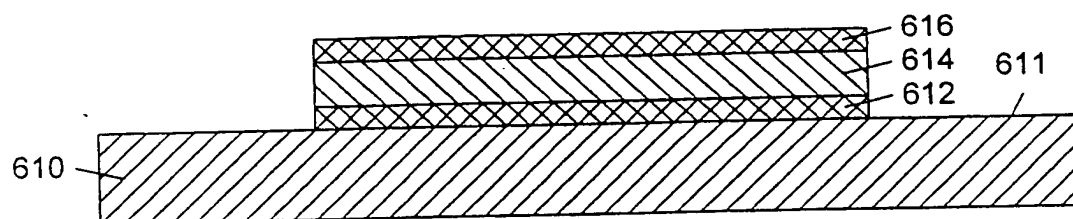


FIG. 23A

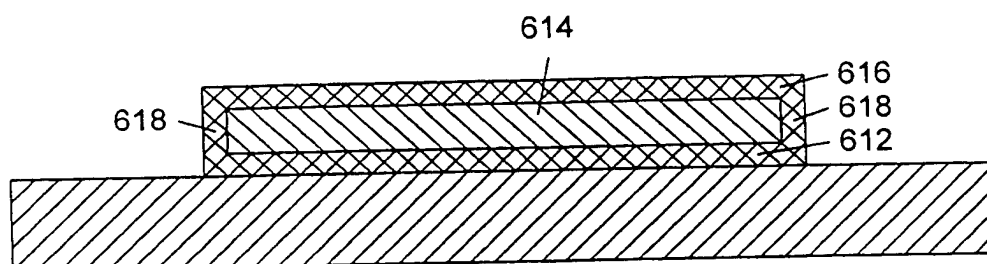


FIG. 23B

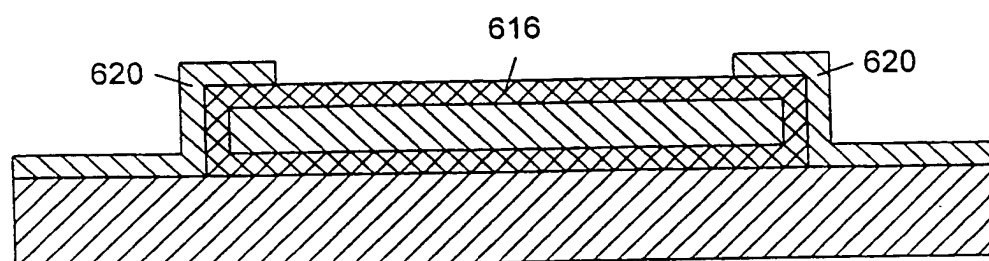


FIG. 23C

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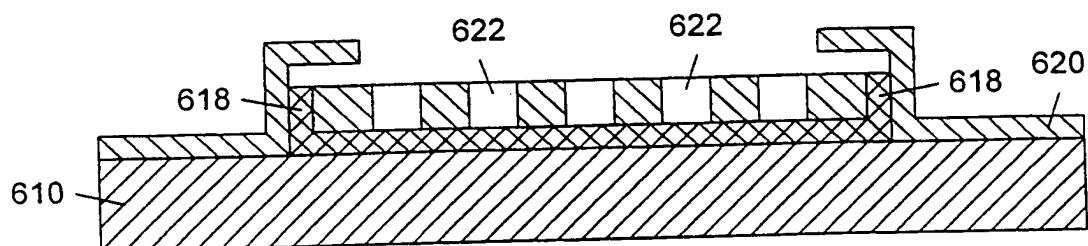


FIG. 23D

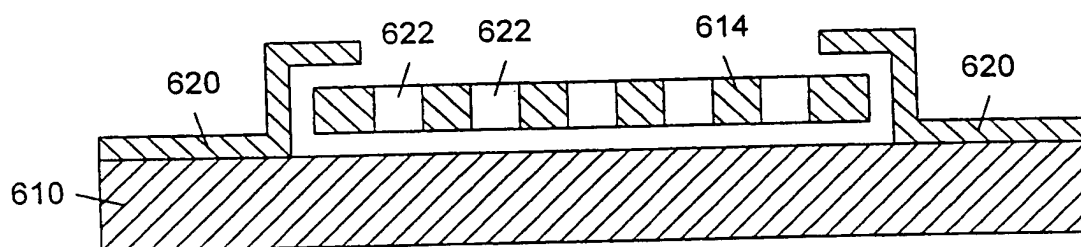


FIG. 23E

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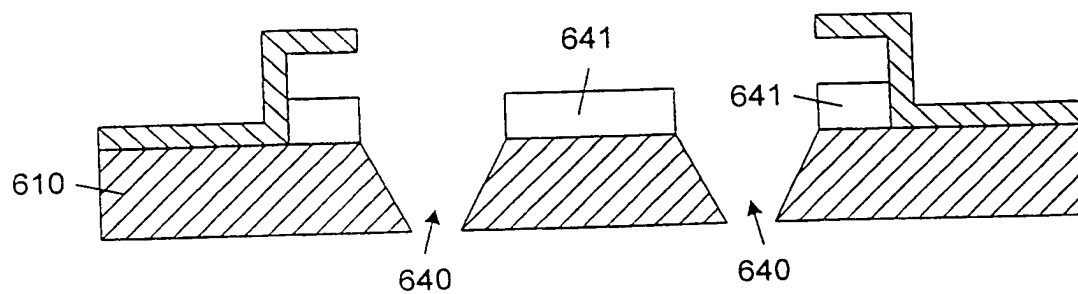


FIG. 23F

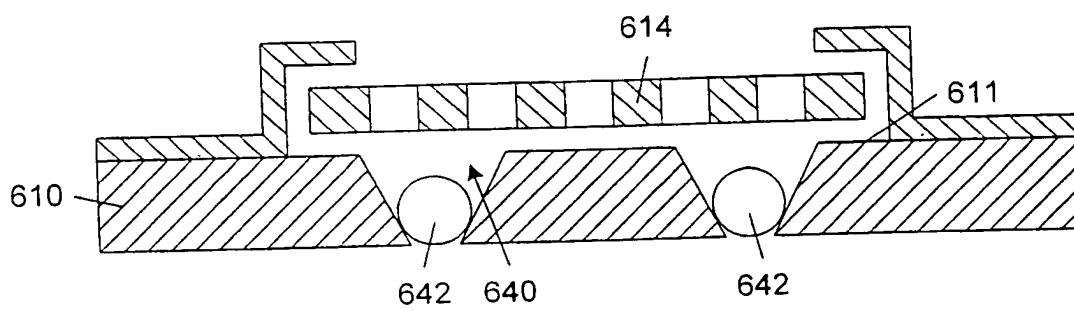


FIG. 23G

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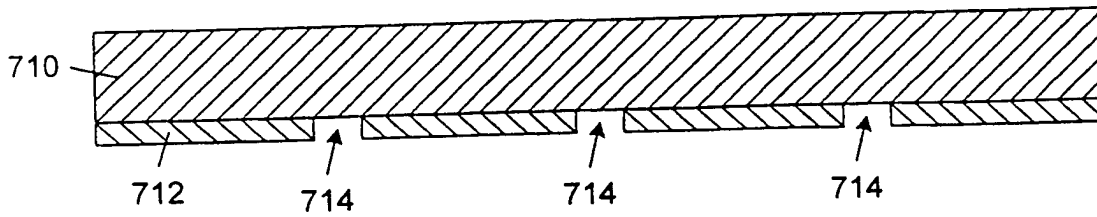


FIG. 24A

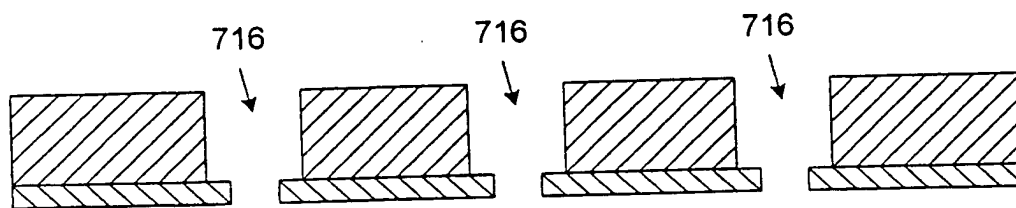


FIG. 24B

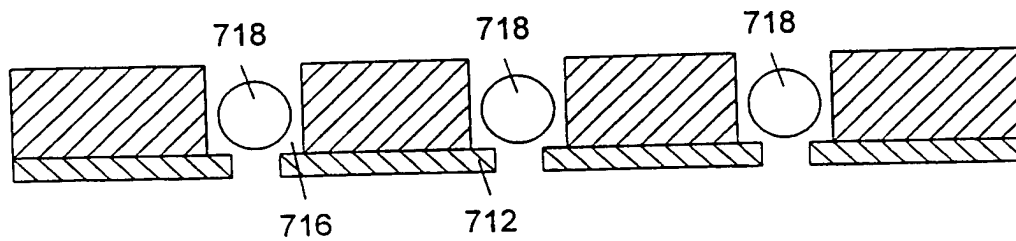


FIG. 24C

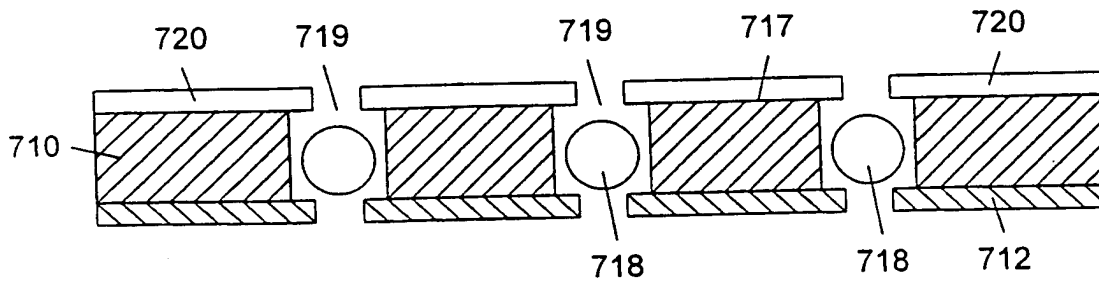


FIG. 24D

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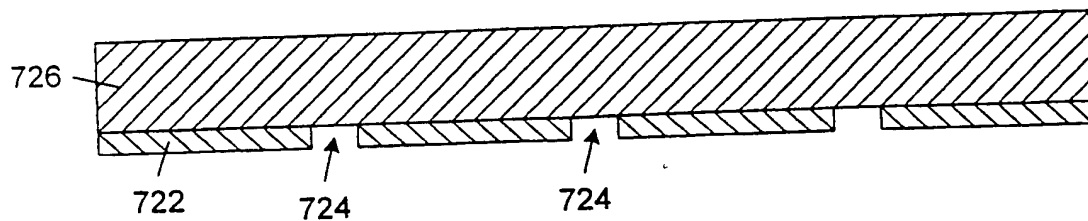


FIG. 25A

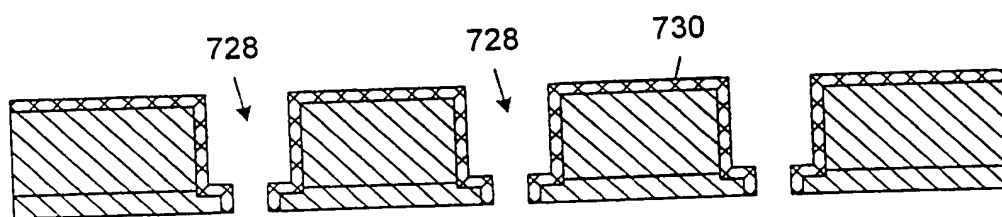


FIG. 25B

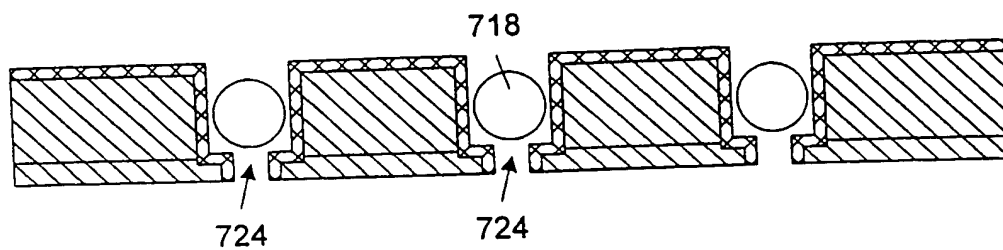


FIG. 25C

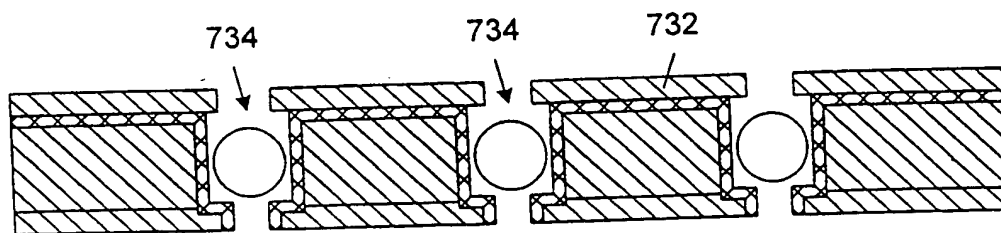


FIG. 25D



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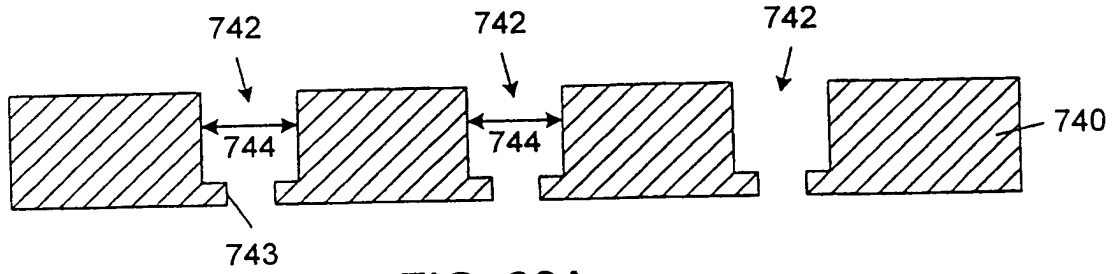


FIG. 26A

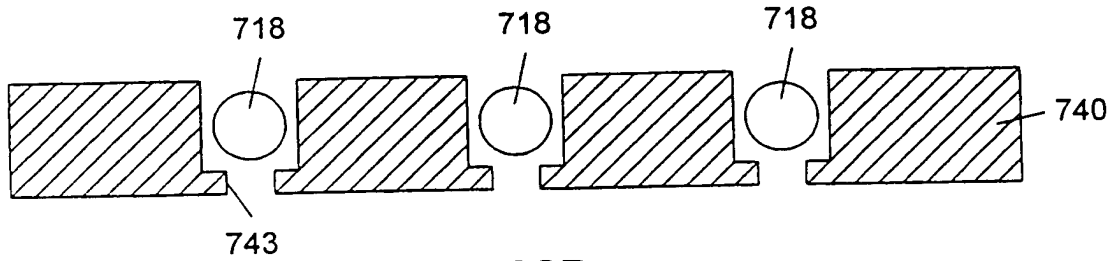


FIG. 26B

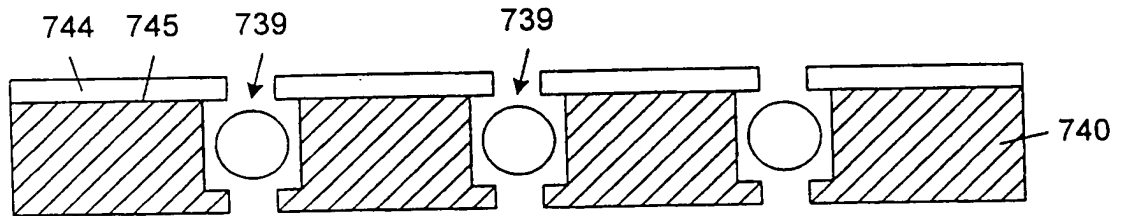


FIG. 26C

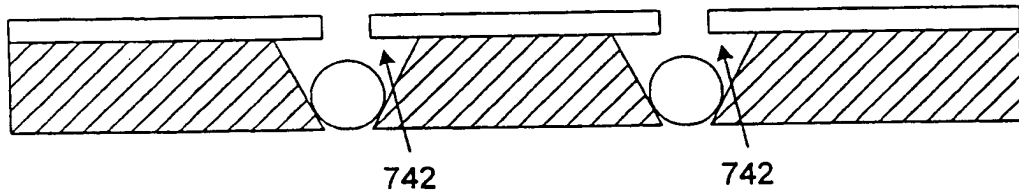


FIG. 26D

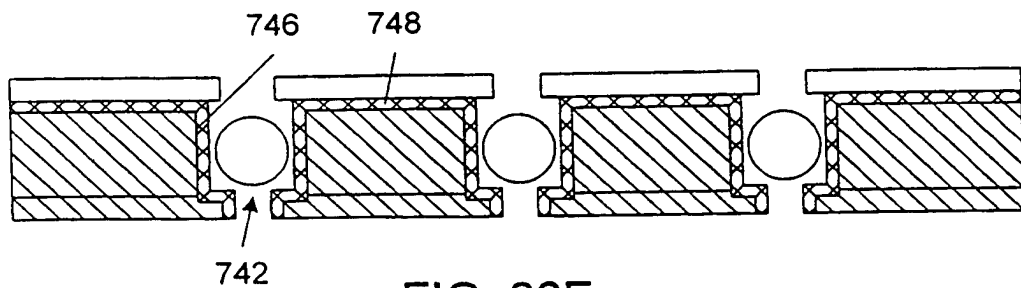


FIG. 26E

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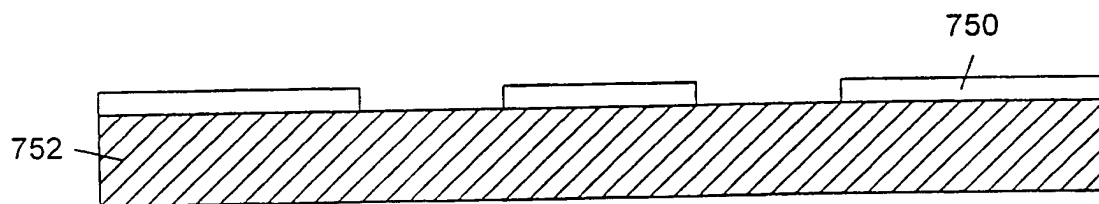


FIG. 27A

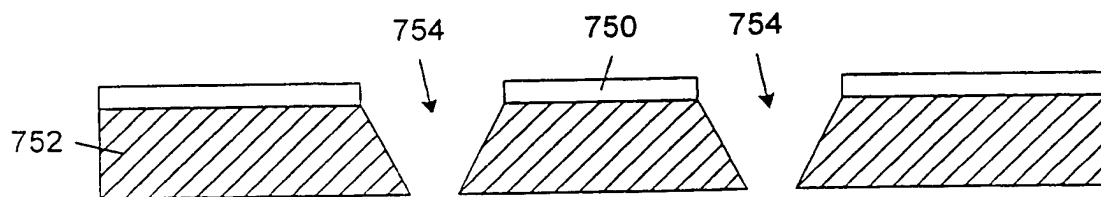


FIG. 27B

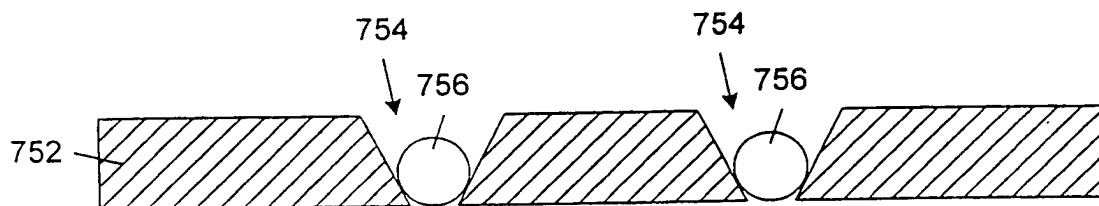


FIG. 27C

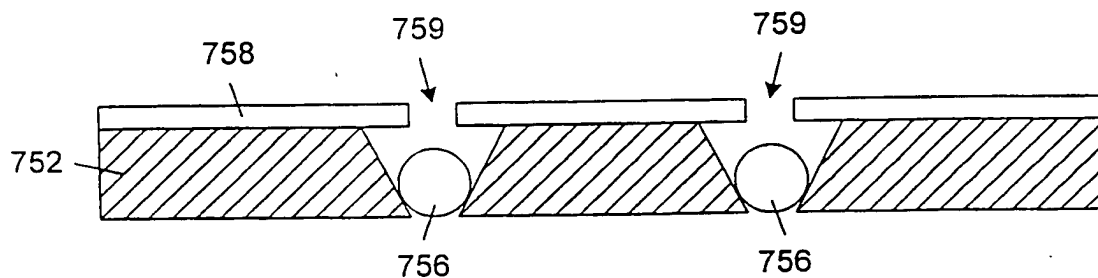


FIG. 27D

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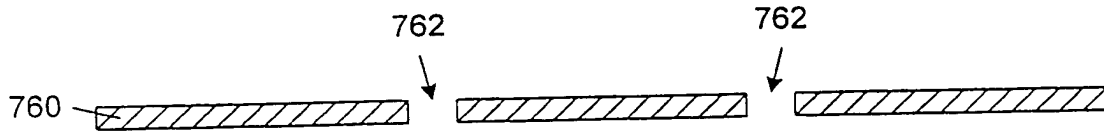


FIG. 28A

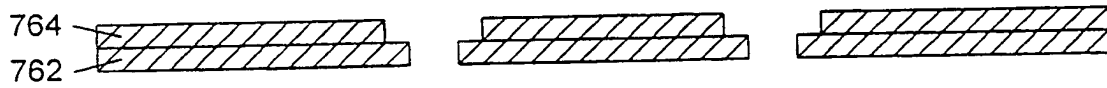


FIG. 28B

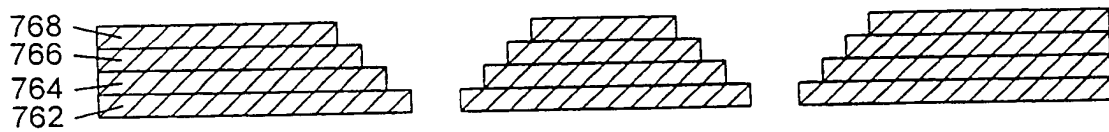


FIG. 28C

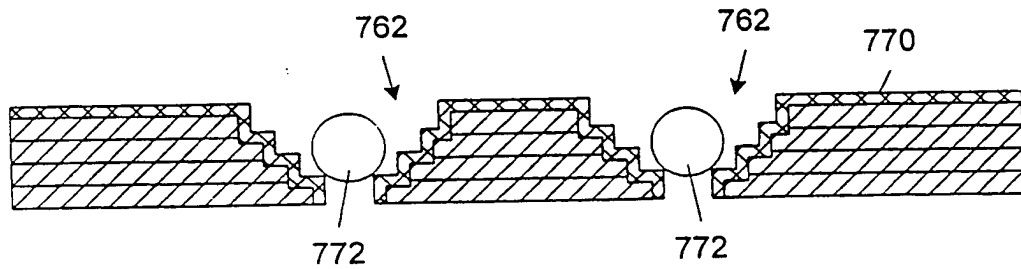


FIG. 28D

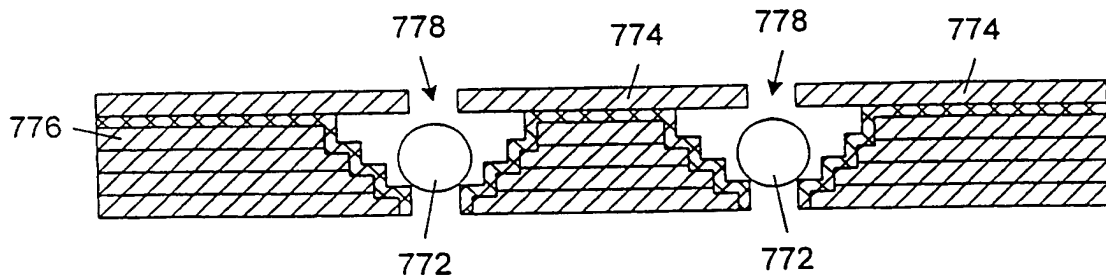


FIG. 28E

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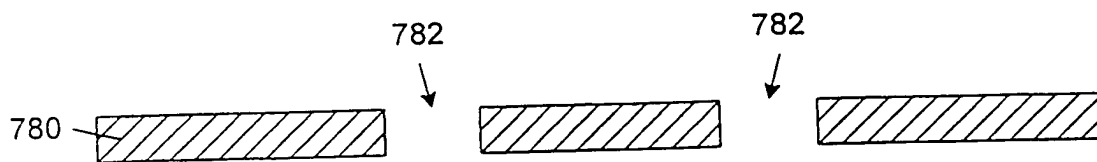


FIG. 29A

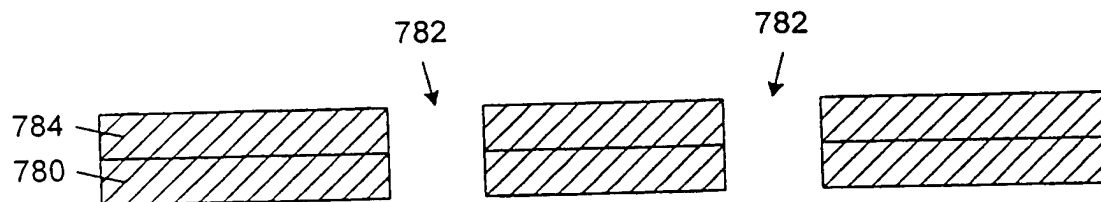


FIG. 29B

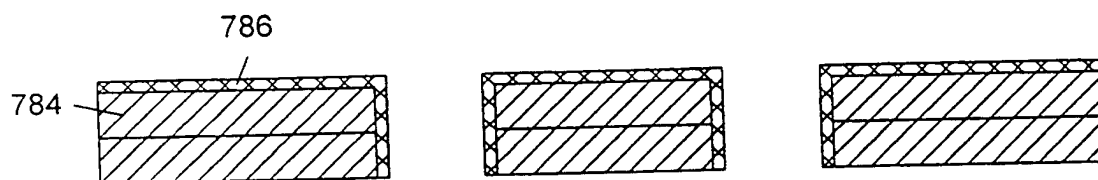


FIG. 29C

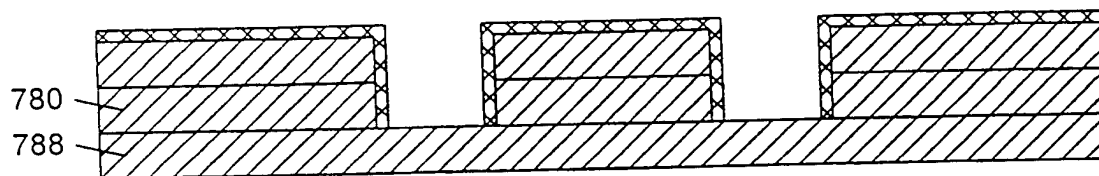


FIG. 29D

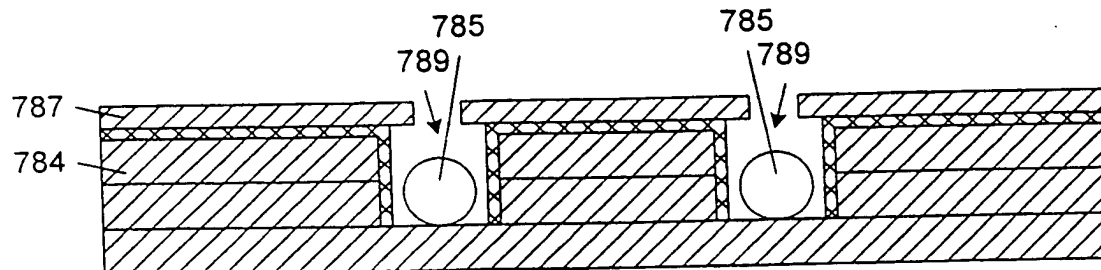


FIG. 29E

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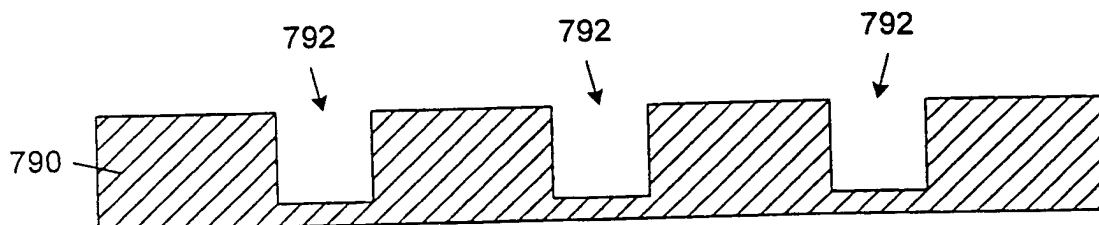


FIG. 30A

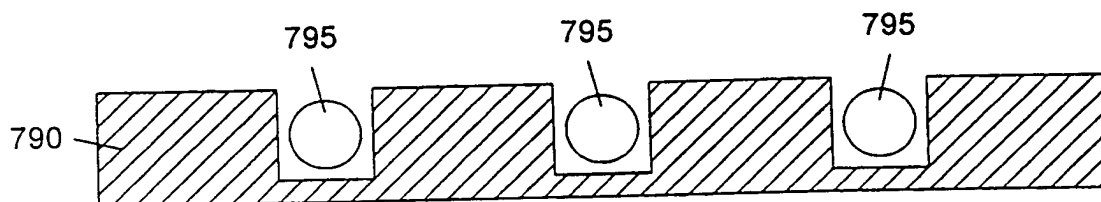


FIG. 30B

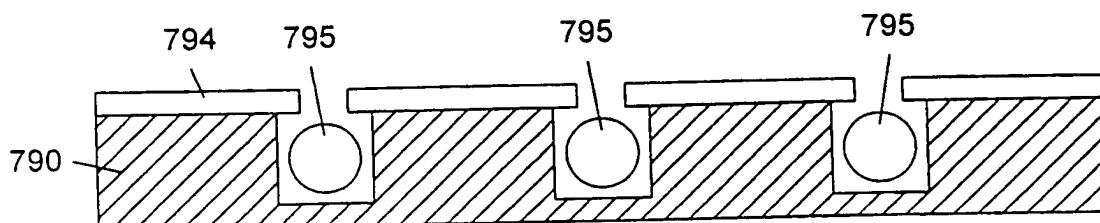


FIG. 30C

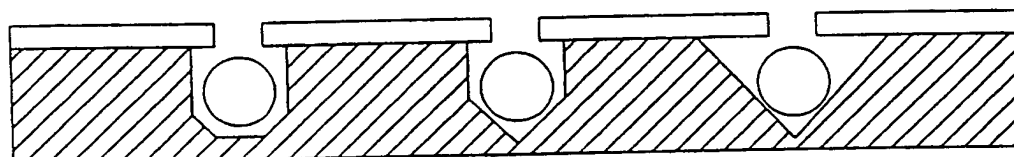


FIG. 30D

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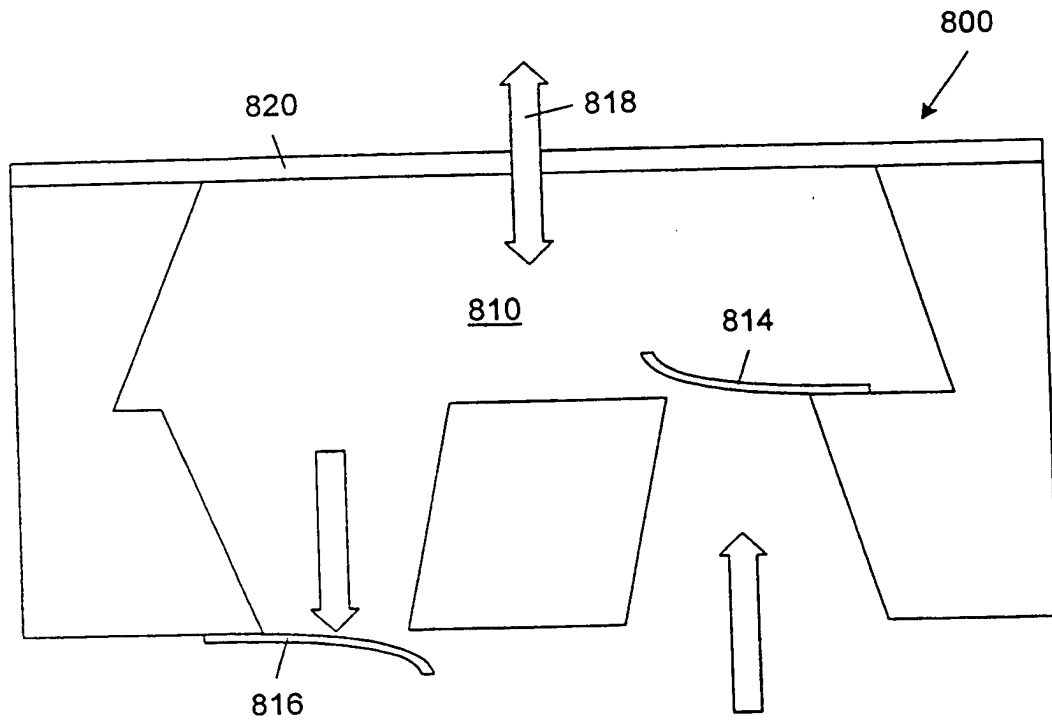


FIG. 31

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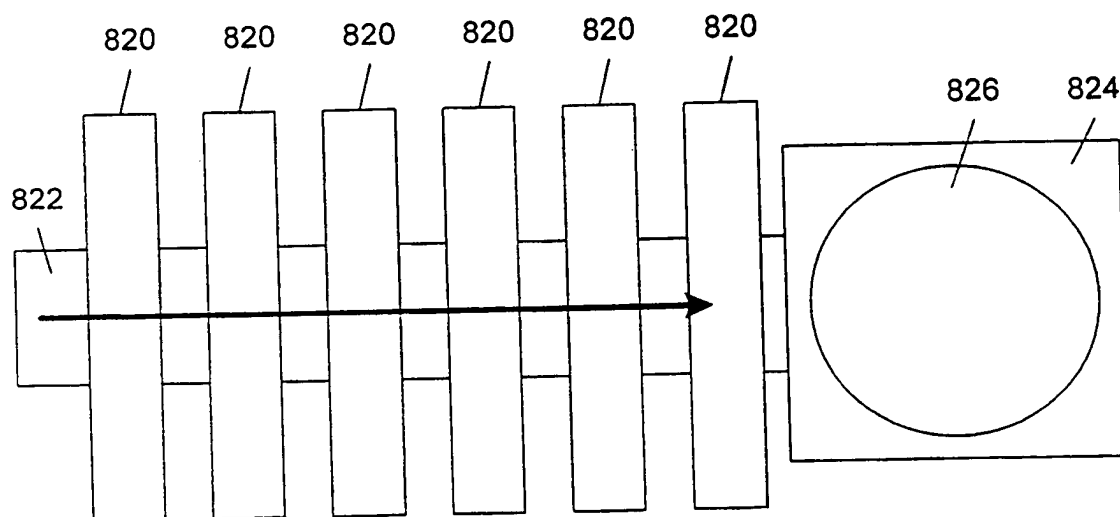


FIG. 32

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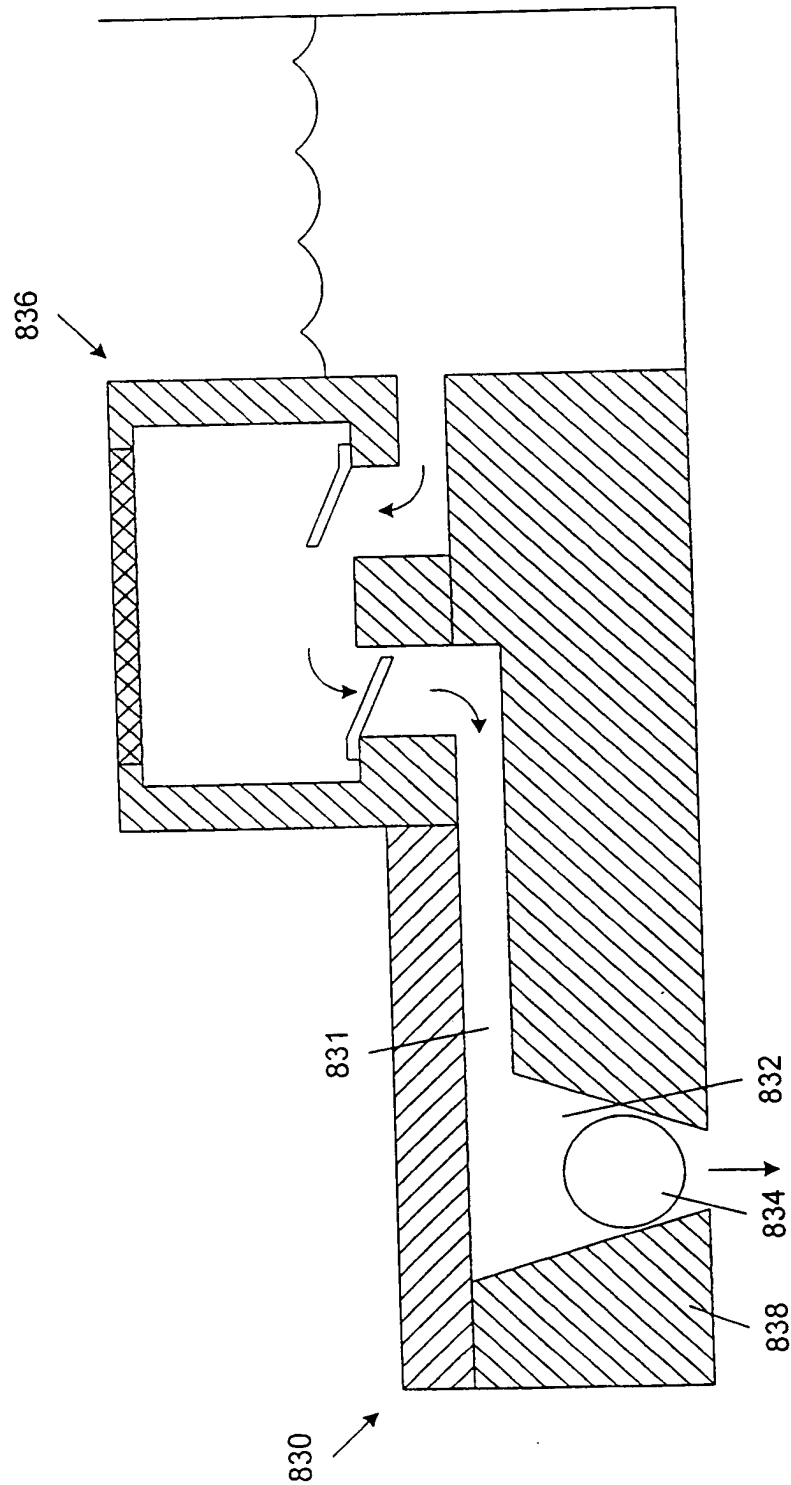


FIG. 33



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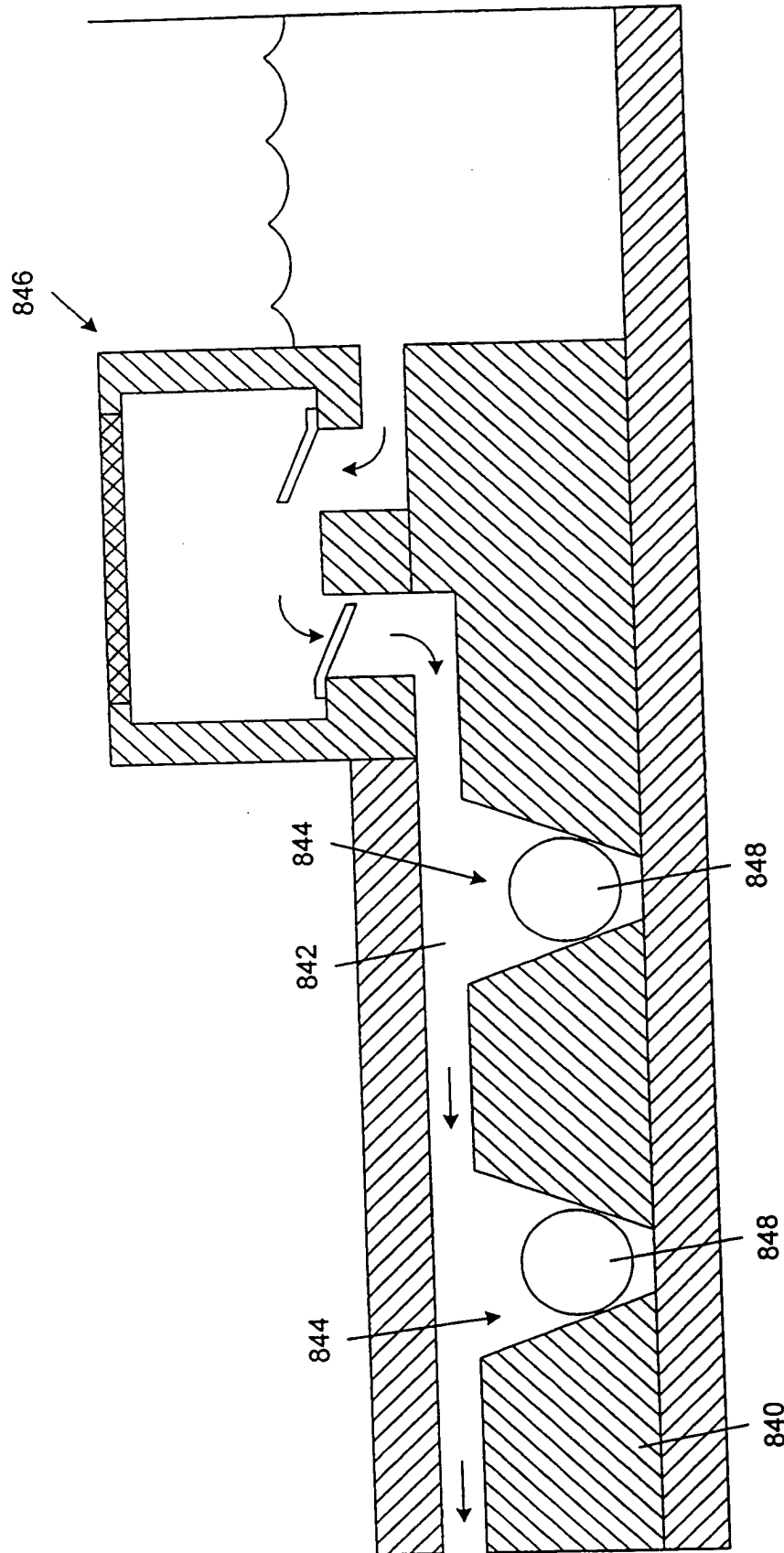


FIG. 34

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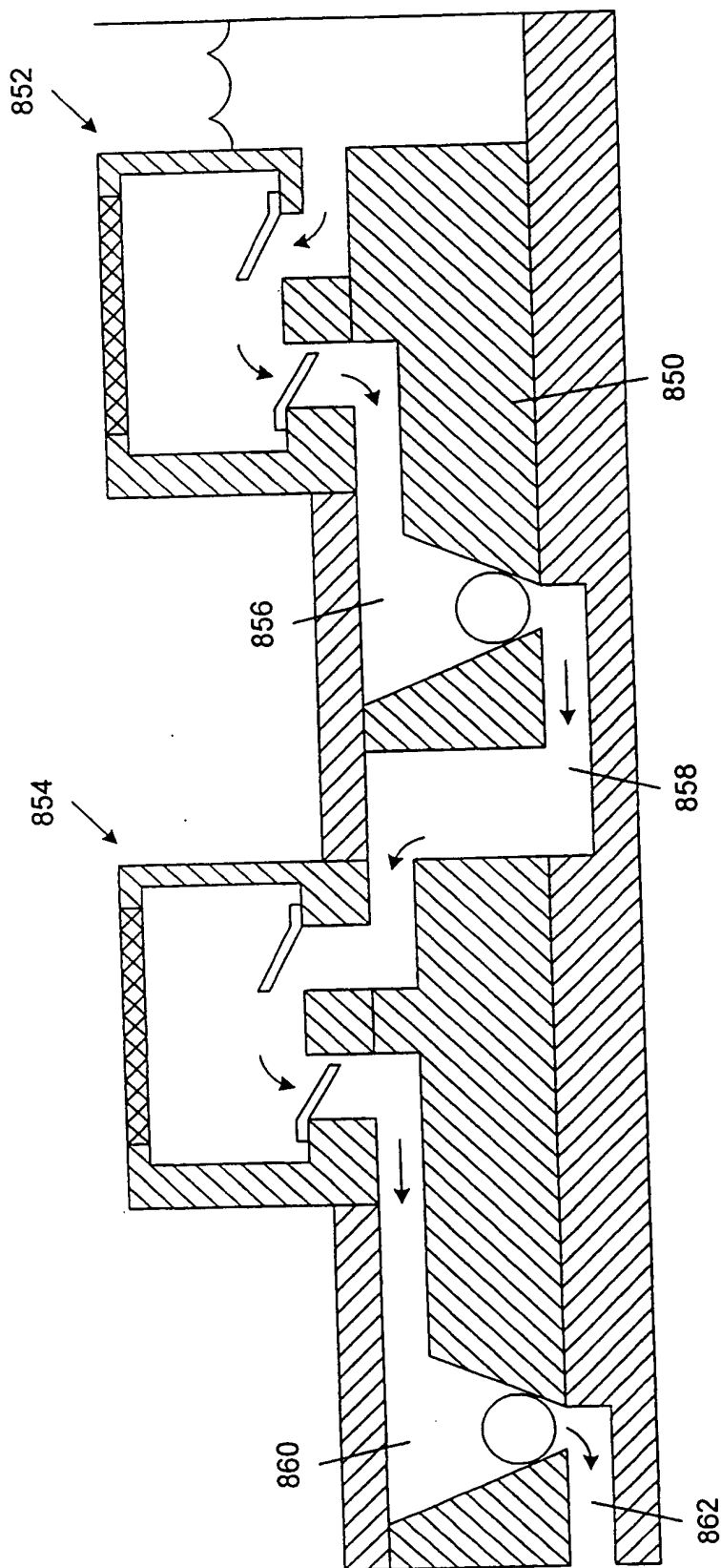


FIG. 35

SUBSTITUTE SHEET (RULE 26)

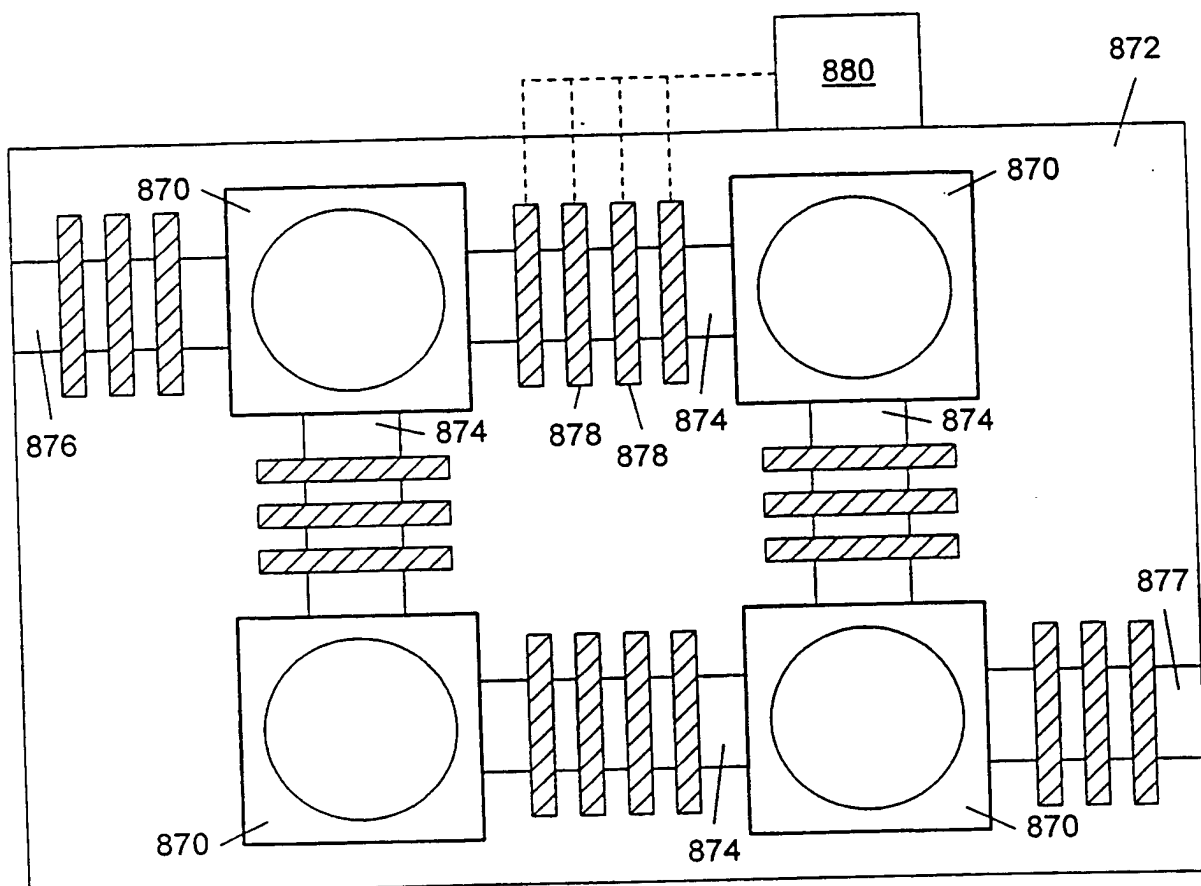


FIG. 36

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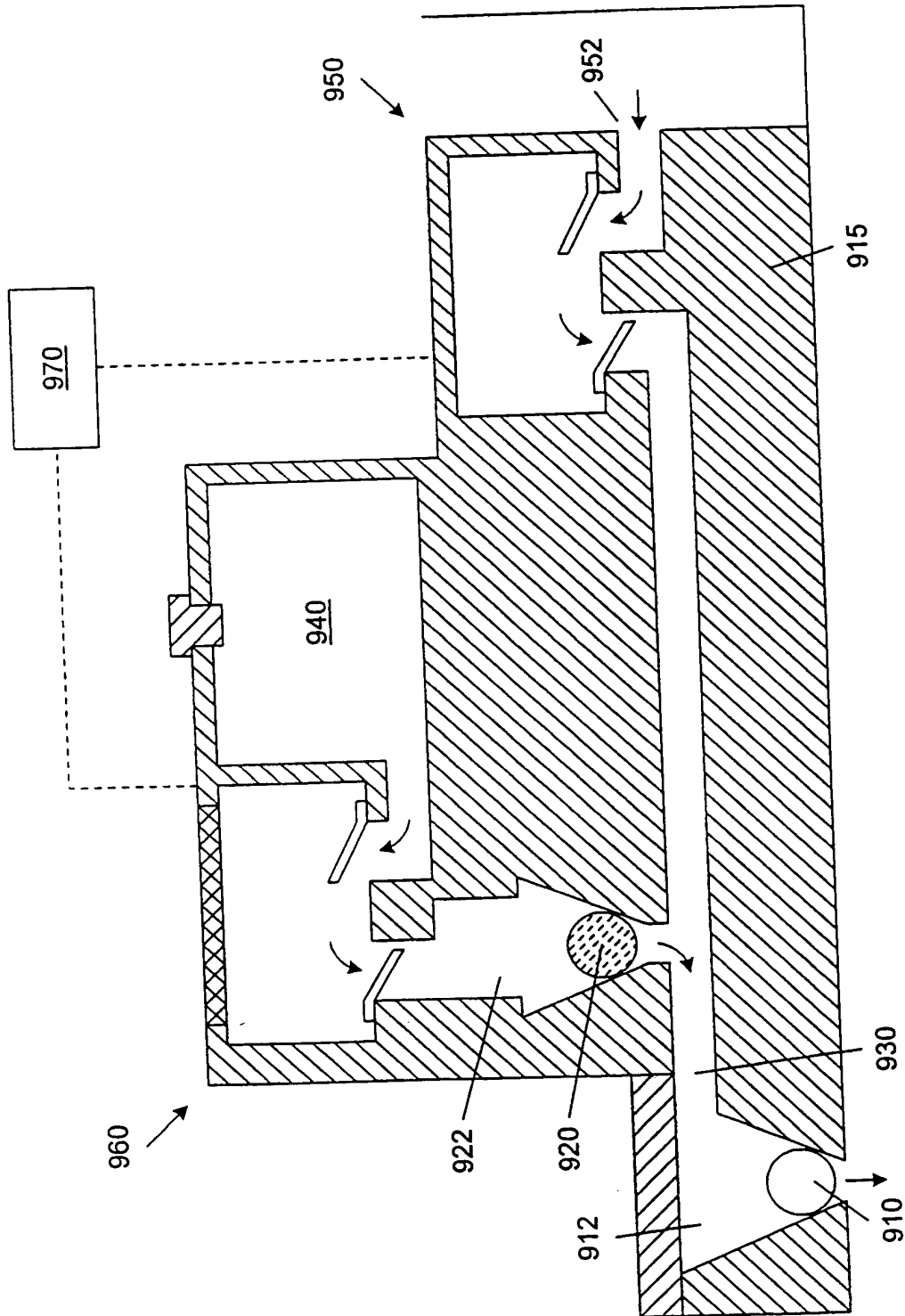


FIG. 37

SUBSTITUTE SHEET (RULE 26)

# INTERNATIONAL SEARCH REPORT

International Application No

F /US 99/16162

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 G01N21/64 G01N21/77

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X.P	<p>WO 99 18434 A (TUFTS COLLEGE ;WALT DAVID R (US); DICKINSON TODD A (US)) 15 April 1999 (1999-04-15)</p> <p>page 8, line 28 -page 9, line 7 page 9, line 13 - line 21 page 10, line 23 - line 35 page 11, line 2 - line 4 page 12, line 5 - line 9 page 13, line 8 - line 17</p> <p>-/--</p>	<p>1-5, 10, 11, 14, 16-20, 23-28, 30-33, 35, 36, 38-42, 48, 49, 51-59, 61-64, 66-74, 84, 95, 106-108</p>

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

18 November 1999

Date of mailing of the international search report

26/11/1999

Name and mailing address of the ISA  
European Patent Office, P.B. 5818 Patentlaan 2  
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Navas Montero, E

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/16162

## C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	<p>page 15, line 11 - line 17  page 16, line 31 - line 38  page 17, line 16 - line 19  page 17, line 23 - line 37  page 19, line 9 - line 16  page 19, line 24 - line 27  page 20, line 22 - line 30  page 21, line 3 - line 12  page 24, line 6 - line 20  page 25, line 24 - line 30  page 26, line 12 - line 18  page 27, line 2 - line 5  page 27, line 6 - line 14  page 29, line 16 - line 18  page 30, line 31 - line 36  table 3  page 33, line 10 - page 34, line 2  page 36, line 28 - line 34  figures 5-8</p> <p style="text-align: center;">---</p>	
X.P	<p>WO 98 53300 A (BRENNER SYDNEY ;LYNX  THERAPEUTICS INC (US); BRIDGHAM JOHN (US);  CO) 26 November 1998 (1998-11-26)</p> <p style="text-align: center;">---</p>	<p>1,6-9,  12,13,  40,  43-47,  50,69,  70,83,  95,96,  105</p>
	<p>page 2, line 6 - line 29  page 3, line 31 - line 36  page 5, line 18 - line 33  page 6, line 1 - line 5  page 6, line 13 - line 17  page 7, line 20 - line 23  page 7, line 27 - line 31  page 10, line 10 - line 14  page 11, line 36 - page 12, line 9  page 22, line 22 - line 28  figures 1-4</p> <p style="text-align: center;">---</p>	
X.P	<p>WO 98 40726 A (TUFTS COLLEGE ;MICHAEL  KARRI C (US); WALT DAVID R (US))  17 September 1998 (1998-09-17)</p> <p style="text-align: center;">---</p>	<p>1,69,  84-86,  89,  93-95,  97,100,  101,  106-108</p>
	<p>page 5, line 18 - line 31  page 6, line 24 - line 27  page 7, line 5 - line 15  page 19, line 15 - page 20, line 26  figures 4-7</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	

## INTERNATIONAL SEARCH REPORT

International Application No

P./US 99/16162

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No
X	WO 97 35189 A (UNIV WASHINGTON) 25 September 1997 (1997-09-25)  page 2, line 19 - line 22 page 9, line 15 - line 18 page 8, line 26 - line 29 page 10, line 19 - line 27 figures 1,4,5  ---	69,75, 76,81, 103
X,P	PATENT ABSTRACTS OF JAPAN vol. 1999, no. 03, 31 March 1999 (1999-03-31) & JP 10 332593 A (HITACHI LTD), 18 December 1998 (1998-12-18) abstract  ---	95,99, 104
X,P	WO 99 17139 A (CLARK HEATHER ;UNIV MICHIGAN (US); BARKER SUSAN (US); KOPELMAN RAO) 8 April 1999 (1999-04-08) page 3, line 23 -page 4, line 2 page 9, line 20 - line 26 figures 1,3  ---	1,40,69
X,P	US 5 866 430 A (GROW ANN E) 2 February 1999 (1999-02-02) column 1, line 11 - line 19 column 1, line 36 - line 46 column 18, line 27 - line 50 column 19, line 54 -column 20, line 11 column 20, line 51 - line 57 column 21, line 17 - line 25 column 21, line 52 - line 60 column 43, line 28 - line 56 column 51, line 25 - line 41 figures 1,5,6  -----	1,40,95

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/ 16162

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 115-412  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.



# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 99 16162

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 115-412

In view of the large number of claims presently on file, which render it difficult, if not impossible, to determine the matter for which protection is sought, the present application fails to comply with the clarity and conciseness requirements of Article 6 PCT (see also Rule 6.1(a) PCT) to such an extent that a meaningful search is impossible. Consequently, the search has been carried out for those parts of the application which do appear to be clear (and concise), namely 1-114.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

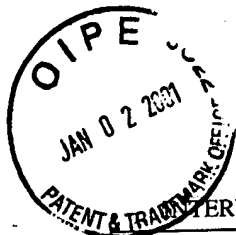
## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

P.../US 99/16162

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9918434	A	15-04-1999	AU	1269599 A	27-04-1999
WO 9853300	A	26-11-1998	AU	7715598 A	11-12-1998
WO 9840726	A	17-09-1998	AU	6464898 A	29-09-1998
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			AU	4000197 A	10-10-1997
JP 10332593	A	18-12-1998	NONE		
WO 9917139	A	08-04-1999	AU	9580798 A	23-04-1999
US 5866430	A	02-02-1999	NONE		



PCT

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International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12P 19/34, C12Q 1/68</b>	<b>A1</b>	(11) International Publication Number: <b>WO 99/67414</b>
		(43) International Publication Date: 29 December 1999 (29.12.99)

(21) International Application Number: PCT/US99/13928

(22) International Filing Date: 22 June 1999 (22.06.99)

(30) Priority Data:

60/090,503	24 June 1998 (24.06.98)	US
60/090,720	26 June 1998 (26.06.98)	US
60/100,703	17 September 1998 (17.09.98)	US
60/108,018	12 November 1998 (12.11.98)	US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications

US	60/090,503 (CIP)
Filed on	24 June 1998 (24.06.98)
US	60/090,720 (CIP)
Filed on	26 June 1998 (26.06.98)
US	60/100,703 (CIP)
Filed on	17 September 1998 (17.09.98)
US	60/108,018 (CIP)
Filed on	12 November 1998 (12.11.98)

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(74) Agents: LEVY, David, J. et al.; Glaxo Wellcome Inc., Five Moore Drive, P.O. Box 13398, Research Triangle Park, NC 27709-3398 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published**

*With international search report.*

*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: NUCLEOTIDE DETECTION METHOD

**(57) Abstract**

Provided is a method of identifying a selected nucleotide in a first nucleic acid utilizing a mobile solid support, as well as a novel read-out method for improving the use of mobile solid support-based read-out technologies for detection of nucleic acid polymorphisms in a target nucleic acid.

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## Nucleotide Detection Method

### Background

#### Field of the Invention

5           The present invention provides methods for rapid detection of single nucleotide polymorphisms (SNPs) in a nucleic acid sample. The present invention further provides a novel read-out method for improving the use of mobile solid support-based read-out technologies for detection of nucleic acid polymorphisms in a target nucleic acid. The methods can be utilized to detect SNPs in genomic DNA as  
10   well as amplified DNA, RNA, etc., thus making them useful for a variety of purposes, including genotyping (such as for disease mutation detection and for parentage determinations in humans and other animals), pathogen detection and identification, and differential gene expression. The present invention further provides a method for identifying a nucleic acid utilizing a run-off sequencing reaction of a relatively short  
15   portion of the nucleic acid. The method can be utilized, for example, to identify an EST from only a small portion of the EST and in an analysis of nucleotide polymorphisms. The reactions can be multiplexed to increase data readout capacity.

#### Background Art

20           Methods of detecting single base polymorphisms have typically involved hybridization reactions. For example, the method of performing a Luminex FlowMetrix-based SNP analysis involves differential hybridization of a PCR product to two differently-colored FACS-analyzable beads. The FlowMetrix system currently consists of uniformly-sized 5 micron polystyrene-divinylbenzene beads stained in  
25   eight concentrations of two dyes (orange and red). The matrix of the two dyes in eight concentrations allows for 64 differently-colored beads ( $8^2$ ) that can each be differentiated by a FACScalibur suitably modified with the Luminex PC computer board. In the Luminex SNP analysis, covalently-linked to a bead is a short

(approximately 18-20 bases) "target" oligodeoxynucleotide (oligo). The nucleotide positioned at the center of the target oligo encodes the polymorphic base. A pair of beads are synthesized; each bead of the pair has attached to it one of the polymorphic oligonucleotides. A PCR of the region of DNA surrounding the to-be analyzed SNP is performed to generate a PCR product. Conditions are established that allow hybridization of the PCR product preferentially to the bead on which is encoded the precise complement. In one format ("without competitor"), the PCR product itself incorporates a fluorescein dye and it is the gain of the fluorescein stain on the bead, as measured during the FACScalibur run, that indicates hybridization. In a second format ("with competitor,") the beads are hybridized with a competitor to the PCR product. The competitor itself in this case is labeled with fluorescein. And it is the loss of the fluorescein by displacement by unlabeled PCR product that indicates successful hybridization. It has been stated that "with competitor" is more discriminating in SNP analysis.

15 A method for typing single nucleotide polymorphisms in DNA, labeled Genetic Bit Analysis (GBA) has been described [Genetic Bit Analysis: a solid phase method for typing single nucleotide polymorphisms. Nikiforov T T; Rendle R B; Goelet P; Rogers Y H; Kotewicz M L; Anderson S; Trainor G L; Knapp M R. NUCLEIC ACIDS RESEARCH, (1994) 22 (20) 4167-75]. In this method, specific fragments of genomic DNA containing the polymorphic site(s) are first amplified by the polymerase chain reaction (PCR) using one regular and one phosphorothioate-modified primer. The double-stranded PCR product is rendered single-stranded by treatment with the enzyme T7 gene 6 exonuclease, and captured onto individual wells of a 96 well polystyrene plate by hybridization to an immobilized oligonucleotide

primer. This primer is designed to hybridize to the single-stranded target DNA immediately adjacent from the polymorphic site of interest. Using the Klenow fragment of *E. coli* DNA polymerase I or the modified T7 DNA polymerase (Sequenase), the 3' end of the capture oligonucleotide is extended by one base using a mixture of one biotin-labeled, one fluorescein-labeled, and two unlabeled dideoxynucleoside triphosphates. Antibody conjugates of alkaline phosphatase and horseradish peroxidase are then used to determine the nature of the extended base in an ELISA format. This paper also describes biochemical features of this method in detail. A semi-automated version of the method, which is called Genetic Bit Analysis (GBA), is being used on a large scale for the parentage verification of thoroughbred horses using a predetermined set of 26 diallelic polymorphisms in the equine genome. Additionally, minisequencing with immobilized primers has been utilized for detection of mutations in PCR products [Minisequencing: A Specific Tool for DNA Analysis and Diagnostics on Oligonucleotide Arrays. Pastinen, T. et al. Genome Research 7:606-614 (1997)].

The effect of phosphorothioate bonds on the hydrolytic activity of the 5'→3' double-strand-specific T7 gene 6 exonuclease in order to improve upon GBA was studied [The use of phosphorothioate primers and exonuclease hydrolysis for the preparation of single-stranded PCR products and their detection by solid-phase hybridization. Nikiforov T T; Rendle R B; Kotewicz M L; Rogers Y H. PCR METHODS AND APPLICATIONS, (1994) 3 (5) 285-91]. Double-stranded DNA substrates containing one phosphorothioate residue at the 5' end were found to be hydrolyzed by this enzyme as efficiently as unmodified ones. The enzyme activity was, however, completely inhibited by the presence of four phosphorothioates. On the

basis of these results, a method for the conversion of double-stranded PCR products into full-length, single-stranded DNA fragments was developed. In this method, one of the PCR primers contains four phosphorothioates at its 5' end, and the opposite strand primer is unmodified. Following the amplification, the double-stranded product is treated with T7 gene 6 exonuclease. The phosphorothioated strand is protected from the action of this enzyme, whereas the opposite strand is hydrolyzed. When the phosphorothioated PCR primer is 5' biotinylated, the single-stranded PCR product can be easily detected colorimetrically after hybridization to an oligonucleotide probe immobilized on a microtiter plate. A simple and efficient method for the immobilization of relatively short oligonucleotides to microtiter plates with a hydrophilic surface in the presence of salt was also described.

DNA analysis based on template hybridization (or hybridization plus enzymic processing) to an array of surface-bound oligonucleotides is well suited for high density, parallel, low cost and automatable processing [Fluorescence detection applied to non-electrophoretic DNA diagnostics on oligonucleotide arrays. Ives, Jeffrey T.; Rogers, Yu Hui; Bogdanov, Valery L.; Huang, Eric Z.; Boyce-Jacino, Michael; Goelet, Philip L.L.C., Proc. SPIE-Int. Soc. Opt. Eng., 2680 (Ultrasensitive Biochemical Diagnostics), 258-269 (1996)]. Direct fluorescence detection of labeled DNA provides the benefits of linearity, large dynamic range, multianalyte detection, processing simplicity and safe handling at reasonable cost. The Molecular Tool Corporation has applied a proprietary enzymatic method of solid phase genotyping to DNA processing in 96-well plates and glass microscope slides. Detecting the fluor-labeled GBA dideoxynucleotides requires a detection limit of approx. 100 mols./ $\mu\text{m}^2$ . Commercially available plate readers detect about 1000 mols./ $\mu\text{m}^2$ , and an



experimental setup with an argon laser and thermoelectrically-cooled CCD can detect approximately 1 order of magnitude less signal. The current limit is due to glass fluorescence. Dideoxynucleotides labeled with fluorescein, eosin, tetramethylrhodamine, Lissamine and Texas Red have been characterized, and  
5 photobleaching, quenching and indirect detection with fluorogenic substrates have been investigated.

Although SNP analysis by hybridization is a powerful method, it has several disadvantages. These include; i) a need to synthesize two targets, and possibly two competitor oligonucleotides for each allelic pair, ii) the establishment of the  
10 hybridization parameters (buffer content, temperature, time) that will efficiently discriminate between alleles, and iii) an avoidance of regions containing secondary structure that may effect the hybridization parameters.

Current limitations to the GBA methods as described include i) the limited density that can be achieved on a 2-dimensional solid surface, ii) photobleaching, iii)  
15 autofluorescence of glass and plastic substrates, iv) difficulty in consistently coupling oligonucleotides to glass, and v) the expense, ease and flexibility of the system for creating new fixed arrays.

The present invention provides a novel system for using a GBA single base chain extension (SBCE) which takes advantage of the powerful matrixing capabilities  
20 of a mobile solid support system having multiple dye color/concentration capabilities (e.g., the FlowMetrix system) to overcome the described disadvantages. The present invention further provides a method to improve the detection of reaction products from such polymorphism identification methods. Various detection methods, as described herein and as known in the art, can be enhanced by utilizing the present

detection method. Such methods can be combined with the present invention to provide a read out format that is time- and cost-efficient as it provides a means of using any given bead for use, individually, with many primers. This read-out method can be utilized also with many polymorphism detection methods, such as SBCE, OLA  
5 and cleavase reaction/ signal release (Invader methods, Third Wave Technologies).

### Detailed Description

The present invention provides a method whereby a mobile solid support, such as a bead, which is detectably tagged, such as with a dye, a radiolabel, a magnetic tag,  
10 or a Quantum Dot® (Quantum Dot Corp.), is utilized in a nucleic acid read out procedure, either a direct readout onto a mobile solid support-linked nucleic acid such as SBCE, OLA or cleavase reaction/signal release (Invader methods, Third Wave Technologies, Madison, Wisconsin) or an indirect readout (in solution) which is then captured by an intermediate nucleic acid such as by a zipcode attached to a mobile  
15 solid support, and the readout product is then analyzed on a selected platform, such as by passing the mobile solid support over a detector (such as a laser detection device) or by passing a detector over the mobile solid support.

The present invention provides a novel system for SNP readout using an encoded mobile solid support which takes advantage of the powerful matrixing  
20 capabilities of a mobile solid support system. In one embodiment, the system uses a GBA single base chain extension (SBCE). In another embodiment, the system utilizes an oligonucleotide ligation assay. In yet another embodiment, the system uses an enzymatic or chemical read-out method whereby an enzyme or chemical is used to modify or endonucleolytically cleave a mismatched base at the polymorphic site,  
25 resulting in the loss of an attached reporter or said modification resulting in a labeling

means for the identification of the modification. Thus, in a further embodiment, the system utilizes an endonuclease cleavase/signal release method (Invader methods, Third Wave Technologies) (see, e.g., Marshall et al. J. Clin. Microbiol. 35(12):3156-3162 (1997); Brow, et al. J. Clin. Microbiol. 34(12):3129-3137 (1997)). In another  
5 embodiment, fluorescence energy transfer (FET) is used with fluorescence quenching as a readout.

In the cleavase enzyme readout, target nucleic acid (e.g., PCR product or genomic DNA) hybridizes to both a complementary Invader probe and a Signal probe; a cleavase enzyme recognizes the specific structure formed between the target  
10 nucleic acid, Invader probe, and Signal probe, and cleaves the Signal probe at the branch site and thereby releases the signal for detection. Another Signal probe can then bind to the nucleic acid and the cleavase reaction begins anew. This process is repeated many times and thereby increases the signal amplification. The essence for cleavase to work is the presence of an overlapping base of the Invader probe with the  
15 signal base. In an improved version, named Invader Squared, two rounds of Invader are performed simultaneously. The primary invader reaction involves using SNP-specific target DNA, the resulting cleavase-product becomes functional in a secondary Invader reaction with a universal signal probe and universal complementary target DNA. After the second round invader assay, a linear signal  
20 amplification of greater than  $10^6$  signal/target/hr is obtained.

The present invention further provides a novel read-out method for improving the use of mobile solid support-based read-out technologies for detection of nucleic acid polymorphisms in a target nucleic acid utilizing a target oligonucleotide having a first complementarity region complementary to the target nucleic acid and a second

complementarity region, 5' to the first complementarity region, complementary to a capture oligonucleotide, which capture oligonucleotide is linked to a mobile solid support. The improved method can be applied to any of several methods of identifying a nucleic acid polymorphism, such as oligonucleotide ligation assay (OLA) or single base chain extension (SBCE), as described herein. The methods can be utilized to detect SNPs in genomic DNA as well as amplified DNA, RNA, etc., thus making it useful for a variety of purposes, including genotyping (such as for disease mutation detection and for parentage determinations in humans and other animals), pathogen detection and identification, and differential gene expression.

10       The present invention further provides the development of a simple method for multiplexing short sequencing reads (about 16 bases) in the same lane. One application to which this method can be applied is high-throughput yeast two-hybrid analysis. In this analysis, it is desired to sequence short regions of the interacting proteins, and then use a large database to determine the hit identification. Because  
15       each bait analyzed generates approximately 100 hits, the present method to increase the efficiency of analysis was needed and therefore developed.

      The invention can be utilized to analyze a nucleic acid sample that comprises genomic DNA, amplified DNA, such as a PCR product, cDNA, cRNA, a restriction fragment or any other desired nucleic acid sample. When one performs one of the  
20       herein described methods on genomic DNA, typically the genomic DNA will be treated in a manner to reduce viscosity of the DNA and allow better contact of a primer or probe with the target region of the genomic DNA. Such reduction in viscosity can be achieved by any desired method, which are known to the skilled artisan, such as DNase treatment or shearing of the genomic DNA, preferably lightly.

Amplified DNA can be obtained by any of several known methods. Sources of genomic DNA are numerous and depend upon the purpose of performing the methods, but include any tissue, organ or cell of choice. Oligonucleotides can be generated by amplification or by de novo synthesis, for example. Complementary nucleic acids, *i.e.*, cRNA (obtained from a process wherein DNA is primed with a T7-RNA polymerase/specific sequence primer fusion, then T7 RNA polymerase is added to amplify the first strand to create cRNA) and cDNA, can be obtained by standard methods known in the art.

Thus, in the present methods, "nucleic acid" includes any of, for example, an oligonucleotide, a 16s ribosomal RNA, a PCR product, a DNA fragment, an RNA molecule, a cDNA molecule or a cRNA molecule, the nucleic acid primer is an oligonucleotide, a PCR product, a DNA fragment, an RNA molecule, a cDNA molecule or a cRNA molecule. Often a primer or a probe in an example is an oligonucleotide, but the source of the primers or probes is not so limited herein.

As used in the claims, "a" and "an" can mean one or more, depending upon the context in which it is used.

In the basic SBCE method, a single oligonucleotide is attached to a detectably tagged, mobile solid support, such as a bead or a rod, preferably that can be processed for detection of the tag quickly once the desired reaction has taken place, such as by a FACS-type system. For example, if one will ultimately fix the support in place prior to detection, a "tentagel" ("octopus") can be used, then fixed in place prior to detection. Any desired tag can be utilized, such as a fluorescent tag, a radiolabel, or a magnetic tag. Other detection systems can be used, preferably, however, wherein the mobile solid support is passed over a detection device, such as a laser detection

device, capable of detecting and discerning the selected tags and labels (*see, e.g.*, PCT publication WO 9714028). Detection systems can also be utilized wherein the mobile solid support, after performing any reactions, is fixed onto a two-dimensional surface and a detection device, such as a laser detection device, is passed over the fixed mobile solid support. The mobile solid support can comprise any useful material, such as polystyrene-divinylbenzene. Detection of the mobile solid support and any nucleic acid or nucleotide associated with it, can be performed by FACS-based method, such as the Luminex FlowMetrix™ system.

In a typical assay, the oligonucleotide is designed such that the 5' end is coupled to the bead. The 3' base ends at a nucleotide chosen relative to the polymorphic base, depending upon the assay being performed. For example, the 3' base of this primer or probe can end at the nucleotide 5' to the polymorphic base, it can end with a base corresponding to the polymorphic base. The length of the oligo in the SBCE method is not critical, but it does need to be long enough to support hybridization by a nucleic acid sample, such as a PCR product generated from a region surrounding the SNP. Depending upon the assay to be performed, the primer or probe can be designed wherein an exact match is required or it may be designed to allow some mismatch upon initial hybridization to the sample nucleic acid.

In a typical assay, a nucleotide capable of chain termination is utilized. Such chain termination is a termination event that occurs before the same labeled base occurs again in the target sequence. Such nucleotides are known in the art and include, for example, a dideoxynucleotide (when polymerase is used in the extension reaction), a thiol derivative (when polymerase is used in the extension reaction), a 3' deoxynucleotide (using reverse transcriptase in the extension reaction), or a 3'

deoxyribonucleotide (using reverse transcriptase in the extension reaction). Any of these nucleotides can be, for example, a dinucleotide, a trinucleotide, or a longer nucleic acid. Thus, one can have, for example, a bank of dinucleotides or longer nucleic acids such that within the bank one has optional nucleotides at more than one  
5 location.

Thus, in the present method, the labeling step is typically performed in solution (thus providing efficient hybridization), and the analysis step can be performed either in solution or on a solid, non-mobile support.

The present invention therefore provides a method of identifying a selected  
10 nucleotide in a first nucleic acid comprising

- (a) contacting the first nucleic acid with a nucleic acid primer linked at its 5' end to a detectably tagged mobile solid support wherein the nucleic acid primer comprises a region complementary to a section of the first nucleic acid that is directly 3' of and adjacent to the selected nucleotide, under hybridization conditions that allow  
15 the first nucleic acid and the nucleic acid primer to form a hybridization product;
- (b) performing a primer extension reaction with the hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension; and
- (c) detecting the presence or absence of a label incorporated into the hybridization  
20 product, the presence of a label indicating the incorporation of the labeled nucleotide into the hybridization product, and the identity of the incorporated labeled nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide in the first nucleic acid.

In a specific embodiment, a primer is designed such that its 3' base ends at the nucleotide immediately 5' of the polymorphic base. A set of 4 dideoxynucleotide triphosphate mixtures are generated. Each mixture contains one of four labeled dideoxynucleotide molecules that have been chemically-coupled to a fluorescein molecule (i.e., ddATP-F, ddCTP-F, ddGTP-F or ddTTP-F), and three non-labeled dideoxynucleotide triphosphates. In one format, the PCR product is added to the bead and the bead aliquoted into 2 or more tubes. The chain-terminating mixtures are dispensed to the tubes and a polymerase is added to generate the SBCE reaction tubes. The polymerase will extend a base onto the 3' end of the bead-attached oligo, this base being the complement of the base at the polymorphic site. The reaction tubes are analyzed by FlowMetrix and the appearance of a label in a particular reaction tube on a particular bead will indicate the polymorphic base at the site.

A comparison of the present method with a hybridization method is illustrative of the utility of the present invention. In the SNP analysis by hybridization, 2 oligos on 2 beads in the same tube are used to generate the material to be read for analysis. In the SCBE method, the same oligo on the same bead is analyzed in 2 tubes with 2 different labeled dideoxynucleotides. Although the method has been exemplified herein using fluorescein as the dye read-out, one can couple this method with biotinylated or other appropriately-modified nucleotides.

The present methods can be performed wherein the chain terminating nucleotide is a dideoxynucleotide and the primer extension is performed in the presence of one labeled, identified dideoxynucleotide and three different, non-labeled dideoxynucleotides. In another embodiment, the chain terminating nucleotide is a dideoxynucleotide, wherein the primer extension is performed in the presence of a



first identified dideoxynucleotide labeled with a first detectable label, a second identified dideoxynucleotide labeled with a second detectable label, a third identified dideoxynucleotide labeled with a third detectable label and a fourth identified dideoxynucleotide labeled with a fourth detectable label, and wherein detection of the presence of the first, the second, the third or the fourth detectable label in the hybridization product indicates the identity of the nucleotide complementary to the selected nucleotide as the first, the second, the third or the fourth dideoxynucleotide, respectively.

Because it is possible to thermal cycle the FlowMetrix beads (Ralph McDade, pers. communication), one can perform a genomic scan using the SBCE method. In this method, the genomic DNA could be sheared, or treated with DNase to reduce viscosity, and cycled against oligos attached to the beads. Because of the vastly greater complexity of the template DNA, it may necessitate the need for extended hybridization optimization and cycling times. Since one would be essentially performing a Cot analysis on the beads. Use of these beads and SBCE for SNP identification and DNA sequencing should be apparent from the above description.

Thus, the present invention provides a method of determining a selected nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising (a) performing an amplification of the genomic DNA using a first nucleic acid primer comprising a region complementary to a section of one strand of the nucleic acid that is 5' of the selected nucleotide, and a second nucleic acid primer complementary to a section of the opposite strand of the nucleic acid downstream of the selected nucleotide, under conditions for specific amplification of the region of the selected nucleotide between the two primers, to form a PCR product;

- (b) contacting the PCR product with a first nucleic acid linked at its 5' end to a detectably tagged mobile solid support, wherein the first nucleic acid comprises a region complementary to a section of one strand of the PCR product that is directly 5' of and adjacent to the selected nucleotide, under hybridization conditions to form a hybridization product;
- (c) performing a primer extension reaction with the hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension;
- (d) detecting the presence or absence of a label incorporated into the hybridization product, the presence of a label indicating the incorporation of the labeled chain-terminating nucleotide into the hybridization product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide; and
- (e) comparing the identity of the selected nucleotide with a non-polymorphic nucleotide, a different identity of the selected nucleotide from that of the non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.
- The PCR product can be in single-stranded form.

The present invention further provides a method of determining a selected nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising

- (a) performing an amplification of the genomic DNA using as a primer an oligonucleotide comprising a first region having a T7 RNA polymerase promoter and a second region complementary to a section of one strand of the nucleic acid that is directly 5' of the selected nucleotide, and using T7 RNA polymerase to amplify one strand into cRNA and using reverse transcriptase to amplify the

- second strand complementary to the cRNA strand, under conditions for specific amplification of the region of the nucleotide between the two primers, to form an amplification product;
- (b) contacting the amplification product with a first oligonucleotide linked at its 5' end to a detectably tagged mobile solid support under hybridization conditions to form a hybridization product;
- (c) performing a primer extension reaction with the hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension;
- (d) detecting the presence or absence of a label incorporated into the hybridization product, the presence of a label indicating the incorporation of the labeled chain-terminating nucleotide into the hybridization product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide; and
- (e) comparing the identity of the selected nucleotide with a non-polymorphic nucleotide, a different identity of the selected nucleotide from that of the non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.
- The labeled chain-terminating nucleotide can be, for example, a 3'deoynucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a dideoxynucleotide. The amplification product can be in single-stranded form.

Furthermore, one can design and synthesize some primers to sit just downstream of the nucleic acids attached to the beads. These can be the primers used to i) make the first strand cDNA, and, ii) with a set that has attached to it the T7 RNA polymerase, can be used to make cRNA. To make the second strand, if needed for the

cRNA, one can use a second primer set that sits outside of the sequence attached to the beads, but just upstream of it. By having the primers off the bead-oligo, they shouldn't interfere by binding. The primers can be made FITC-labeled for the cDNA.

- The present method further provides a method of determining a selected
- 5 nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising
- (a) contacting the genomic DNA with a first primer linked at its 5' end to a detectably tagged mobile solid support, wherein the first primer comprises a first region complementary to a section of one strand of the genomic DNA that is directly 5' of and adjacent to the selected nucleotide under hybridization conditions for
- 10 forming a specific hybridization product;
- (b) performing a primer extension reaction with the specific hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension;
- (c) detecting the presence or absence of a label incorporated into the hybridization
- 15 product, the presence of a label indicating the incorporation of the labeled chain-terminating nucleotide into the hybridization product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide; and
- (d) comparing the identity of the selected nucleotide with a non-polymorphic
- 20 nucleotide, a different identity of the selected nucleotide from that of the non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.

The DNA can be in single-stranded form. The labeled chain-terminating nucleotide can be, for example, a 3'deoynucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a dideoxynucleotide. In such a method, the hybridization time should be

of a length sufficient to allow hybridization of the first primer to the genomic DNA since the genomic DNA has not been amplified in this specific embodiment. Thus relatively long hybridization times may be utilized, such as, for example, 12 hours, 24 hours, 48 hours, as is known in the art for hybridization to genomic DNA (*see, e.g.,* 5 Sambrook, *et al. Molecular Cloning: A Laboratory Manual*, 2d ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 1989).

For any of the herein described reactions, alternative polymerases can be employed, such as a polymerase with a temperature condition for function, or a polymerase with a particular specificity for nucleotides, such as a polymerase that 10 preferentially incorporates dideoxynucleotides (*see, e.g.,* Sambrook, *et al.*). The skilled artisan is familiar with such polymerases, and new polymerases, as they are discovered, can be incorporated into the methods, given the teachings herein..

The present invention additionally provides the use of the beads in an oligonucleotide-ligation assay (OLA) format, i.e., in which one can hybridize 15 genomic DNA, cRNA or PCR product to a first nucleic acid attached to the bead, then come in with a second nucleic acid with a fluorescent label, then add ligase, and wherein the second nucleic acid has at its 3' end the polymorphic bases. Thus, the present invention provides a method of identifying a selected nucleotide in a first nucleic acid comprising

20 (a) contacting the first nucleic acid with (i) a second nucleic acid linked at its 5' end to a detectably tagged mobile solid support wherein the second nucleic acid comprises a region complementary to a section of the first nucleic acid that is directly 3' of the selected nucleotide and wherein the second nucleic acid terminates at its 3' end in a test nucleotide positioned to base-pair with the selected nucleotide, and (ii) a third,

fluorescently labeled nucleic acid, wherein the third nucleic acid comprises a region complementary to a section of the second nucleic acid that is directly 5' of and adjacent to the selected nucleotide,

under hybridization conditions that allow the first nucleic acid and the second  
5 nucleic acid to form a hybridization product and the first nucleic acid and the third nucleic acid to form a hybridization product;

(b) adding to the hybridization product a ligase under ligation conditions; and

(c) detecting the presence or absence of the fluorescent label, after dissociation of the  
hybridized nucleic acids, in the nucleic acid linked to the mobile solid support,  
10 the presence of the label indicating the ligation of the labeled third nucleic acid to the second nucleic acid linked to the mobile solid support, and the identity of the test nucleotide in the second nucleic acid indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide.

This oligonucleotide ligation assay can be performed both (a) wherein the  
15 polymorphic base is located at the 5' side of either the reporter or acceptor oligonucleotide, or (b) wherein the polymorphic base is located at the 3' side of either the reporter or acceptor oligonucleotide.

The first nucleic acid can be genomic DNA (treated to reduce viscosity, e.g., by DNase treatment or by shearing), amplified nucleic acid such as a PCR product, an  
20 oligonucleotide, a 16s ribosomal RNA, a DNA fragment, an RNA molecule, a cDNA molecule, a cRNA molecule, restriction enzyme-generated DNA fragment, size-selected DNA, Bridge-amplified DNA, 16S RNA, 16S DNA or any other desired nucleic acid.. Any selected ligase can be used, such as T4 DNA ligase. A

thermostable ligase would be particularly useful. *See, generally* Wu and Wallace, Genomics 4: 560-569 (1989).

The present invention additionally encompasses the use in the OLA readout of degenerate reporter oligonucleotides, preferably the use of 8-mer oligonucleotides wherein 6 of the bases are chosen to be specific to the target nucleic acid and 2 of the bases are variable, or wobble or degenerate, positions. The degeneracy can be placed in any position in the reporter oligonucleotide; however, preferable positions can be positions 3, 4, 5, and 6. Preferable variable position combinations in a selected oligonucleotide can be positions 3 and 6, positions 4 and 5, and positions 3 and 4.

Thus, one can synthesize all possible "6+2-mers" as reagents for use in an assay, whereas synthesis of all possible 8-mers is not practicable. Furthermore, non-natural derivatives, such as inosine, can be utilized in the reporter oligonucleotides. For example, the present invention includes an OLA readout wherein the reporter oligonucleotide is an 8-base complementary 8-mer conjugated to a reporter molecule or hapten to which a reporter molecule can be conjugated by means of a hapten-recognizing intermediary (e.g., antibody, avidin, streptavidin). The present invention further includes an OLA readout wherein the reporter oligonucleotide is an 6-base complementary 8-mer ("6+2-mer") conjugated to a reporter molecule or hapten to which a reporter molecule can be conjugated by means of a hapten-recognizing intermediary. The two non-complementary bases can be any of the four natural bases or can be a non-natural derivative capable of forming a non-helix disturbing duplex structure. The non-complementary bases can preferably be located at positions 3 and 6 or positions 4 and 5. Non-natural base derivatives and/or 6+2-mers can be components of a kit for use in performing the detection methods described herein.

Further, one can employ a 'Taqman' approach wherein one incorporates Dye quenchers and Dye acceptors into the attached oligos and asks for the polymerase to remove the dye quencher in a repair reaction.

The invention further employs hybridization methods wherein two  
5 nucleic acids are hybridized to the sample nucleic acid but the step of ligation can be omitted and a match instead detected by fluorescence energy transfer between the two nucleic acids hybridized to the sample nucleic acid. The two hybridizing nucleic acids are designed such that the 3' end of the nucleic acid linked to the bead is a test base, and when it is complementary to the polymorphic base, and a single wavelength  
10 of light is directed onto the sample, one can detect a transfer of energy, read as a second wavelength of light. A second reader can be employed for this detection of this second wavelength. Thus, the present invention provides a method of identifying a selected nucleotide in a first nucleic acid comprising

(a) contacting the first nucleic acid with

- 15 (i) a second nucleic acid linked at its 5' end to a detectably tagged mobile solid support and linked at its 3' end to a fluorescent label, wherein the second nucleic acid comprises a region complementary to a section of the first nucleic acid that is directly 3' of the selected nucleotide and wherein the second nucleic acid terminates at its 3' end in a test  
20 nucleotide positioned to base-pair with the selected nucleotide, and
- (ii) a third nucleic acid fluorescently labeled at its 5' end, wherein the third nucleic acid comprises a region complementary to a section of the second nucleic acid that is directly 5' of and adjacent to the selected nucleotide,



under hybridization conditions that allow the first nucleic acid and the second nucleic acid to form a hybridization product and the first nucleic acid and the third nucleic acid to form a hybridization product; and

(c) detecting the presence or absence of fluorescent energy transfer between  
5 the fluorescent label at the 3' end of the second nucleic acid and the fluorescent label at the 5' end of the third nucleic acid, the presence of fluorescent energy transfer indicating the hybridization of the test nucleotide to the first nucleic acid, and the identity of the hybridized test nucleotide in the second nucleic acid indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying  
10 the selected nucleotide. The detection of the fluorescence energy transfer (FET) can be performed after dissociation of the hybridized nucleic acids.

The present invention also provides a method for determining the sequence of a polymorphic base comprising: a first nucleic acid attached at a 5' end to a mobile solid support and having a 3' end adjacent to a polymorphic base on a second nucleic acid;  
15 a third nucleic acid with an attached reporter moiety that is complementary to a region adjacent to the polymorphic base of the second nucleic acid; the first nucleic acid and the third nucleic acid together defining a gap opposite the polymorphic base; a nucleotide that is complementary to one of a set of two possible polymorphic bases, a polymerase, and a ligase; wherein the polymerase is able to polymerize the nucleotide  
20 across the gap if the nucleotide is complementary to the polymorphic base; the ligase is able to ligate the newly polymerized nucleotide to the reporter-attached third nucleic acid; and a means for detecting the reporter covalently linked to the bead. Specifically, the present invention provides a method of identifying a selected nucleotide in a first nucleic acid comprising

- (a) contacting the first nucleic acid with
- (i) a second nucleic acid linked at its 5' end to a detectably tagged mobile solid support, wherein the second nucleic acid comprises a region complementary to a section of the first nucleic acid that is directly 3' of and immediately adjacent to the selected nucleotide, and
  - (ii) a third nucleic acid fluorescently labeled, wherein the third nucleic acid comprises a region complementary to a section of the second nucleic acid that is directly 5' of and adjacent to the selected nucleotide,
- under hybridization conditions that allow the first nucleic acid and the second nucleic acid to form a hybridization product and the first nucleic acid and the third nucleic acid to form a hybridization product, wherein the first, second and third nucleic acids form a hybridization product that defines a gap opposite the selected nucleotide;
- (b) adding a test nucleotide, a polymerase and a ligase, under conditions for polymerization and ligation; and
- (c) detecting the presence or absence of the fluorescent label, after dissociation of the hybridized nucleic acids, in the nucleic acid linked to the mobile solid support, the presence of the label indicating the polymerization of the test nucleic acid to the second nucleic acid and ligation of the labeled third nucleic acid to the second nucleic acid linked to the mobile solid support, and the identity of the test nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide.

The polymerase can preferably be a non-strand displacing polymerase. Further, it can be a thermostable polymerase. The ligase can be a DNA ligase. Further, it can be a thermostable ligase.

The present invention further provides a method of detecting a single base polymorphism comprising using an enzyme or chemical to modify or endonucleolytically cleave a mismatched base at the polymorphic site in a nucleic acid, resulting in the loss of an attached reporter or in a modification, and detecting a loss of the reporter or detecting the modification, thus resulting in a labeling means for the identification of the modification. In one example, an end-labeled (such as with FITC) genomic fragment or a labeled (such as with FITC) PCR fragment is hybridized to an oligonucleotide and attached to a bead, then the construct is treated with an enzyme that recognizes and/or restricts mispaired DNA (such as FITC-labeled recA, mutS or T7 enzyme) and analyzed for the addition or loss of the label. In another example, a chemical recognizing single stranded regions of DNA and capable of modifying the region is utilized, and the modification is detected.

Furthermore, any of the herein described methods can be utilized in a method for quantitating expression of a selected nucleic acid in a sample. Thus, it can be used, for example, for differential gene expression wherein the expression of a selected gene is quantitated and compared to a standard or some other reference. For this method, a gene fragment from a region of interest or a region that distinguishes the gene (or allele or haplotype or polymorphism) of interest is linked at its 5' end to a detectably labeled mobile solid support; message (e.g., RNA, cDNA, cRNA) is hybridized to the fragment, and fluorescence is quantitated by performing a primer extension reaction, a ligase reaction or a hybridization/fluorescence energy transfer

reaction, such as that described herein. The nucleic acid probe can comprise a region complementary to a section of the selected nucleic acid unique to the nucleic acid. A standard, such as that from a normal subject, or a diseased/ afflicted subject, or a particular tissue or organ, or a particular species, can be used as a comparison  
5 reference to draw conclusion regarding the quantity detected in the sample.

Specifically, the present invention provides a method of detecting a result from an identification reaction to identify a selected nucleotide in a target nucleic acid comprising:

- 10 a) contacting a target oligonucleotide comprising a first complementarity region and a second complementarity region, wherein the second complementarity region is 5' of the first complementarity region and wherein the first complementarity region comprises a region complementary to a section of the target nucleic acid that is directly 3' of and adjacent to the selected nucleotide, with a sample comprising the  
15 target nucleic acid, under hybridization conditions that allow the formation of a hybridization product between the first complementarity region of the target oligonucleotide and a region of the target nucleic acid complementary to the first complementarity region of the target oligonucleotide, to form a first hybridization product;
- 20 b) performing a selected identification reaction with the first hybridization product to determine the identity of the selected nucleotide wherein a selectively labeled detection product comprising the second complementarity region of the target oligonucleotide can be formed;

- c) isolating the detection product by contacting the detection product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form a second hybridization product; and
- d) detecting and/or identifying the label of the labeled detection product in the second hybridization product,
- the presence and or identity of the label indicating the identity of the selected nucleotide in the target nucleic acid.

The basic method thus involves the use of a capture oligonucleotide, linked to a mobile solid support (such as a bead), to isolate a reaction product from a reaction. To facilitate this isolation, a "target oligonucleotide" is designed which comprises, in addition to a first complementarity region, which is a region complementary to a region of the target nucleic acid, a second complementarity region, which is located 5' of the first complementarity region, and which is complementary to the nucleotide sequence of the capture oligonucleotide. Thus, before or after a reaction (such as SBCE or OLA), the capture oligonucleotide can be utilized in a hybridization reaction to isolate the target oligonucleotide in its reacted form (e.g., as a ligation product or as a primer extension product). Thus, one is not obligated, as in many other assays, to synthesize a bead specifically for each oligonucleotide (e.g., the "first complementarity region of the target oligonucleotide in the present invention) that is to be hybridized to the target nucleic acid.

The present invention additionally encompasses the use in the OLA readout of degenerate reporter oligonucleotides, preferably the use of 6+2-mers as described herein. Such reporter oligonucleotides can be a component of a useful kit for performing the detection methods herein.

5       The capture oligonucleotide can be designed such that it does not specifically hybridize, *i.e.*, is not sufficiently complementary for specific hybridization to occur, to the target nucleic acid. For example, it can include nucleotide usage not typically found in the target species (such as human). If the target sequence is fully known, the capture sequence can be selected as a sequence which does not occur in the target  
10       sequence. A capture oligonucleotide can be of any desired length so long as it is sufficiently long so as to selectively hybridize to a first complementarity region of a target oligonucleotide (under selective hybridization conditions, *e.g.*, stringent hybridization conditions, as known to one skilled in the art), and not so long as to interfere with either the identification reaction being performed with the target  
15       oligonucleotide or the hybridization reaction between the capture oligonucleotide and the target oligonucleotide. The capture oligonucleotide length selected can also be a function of how many different capture oligonucleotides one desires to use in any selected use. For example, the capture oligonucleotide can be 8, 10, 12, 15, 18, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40 or more nucleotides. A preferred length is  
20       around 25 nucleotides, such as 23, 24, 25, 26, 27 or 28 nucleotides. However, other oligonucleotide lengths can be utilized. Optimal length for any specific use can be determined according to the specific nucleic acid composition, as will be known to those skilled in the art.

One can advantageously create a bank of several capture oligonucleotides, each linked to a different color of bead. A bank of complementary regions can be maintained for use in generating target oligonucleotides for any specific target nucleic acid. Thus, one can utilize a defined set of beads, and simply create new target  
5 nucleotides as necessary for any given detection task.

The present invention provides a method of detecting a reaction product to identify a selected nucleotide in a target nucleic acid comprising:

- a) contacting a target oligonucleotide comprising a first complementarity region and a second complementarity region, wherein the first  
10 complementarity region comprises the oligonucleotide primer and the second complementarity region comprises a nucleic acid sequence complementary to a capture oligonucleotide, and wherein the oligonucleotide primer comprises a region complementary to a section of the target nucleic acid that is directly 3' of and adjacent to the selected  
15 nucleotide, with a sample comprising the target nucleic acid, under hybridization conditions that allow the formation of a hybridization product between the first complementarity region of the target oligonucleotide and a region of the target nucleic acid complementary to the first complementarity region of the target oligonucleotide, to form a  
20 first hybridization product;
- b) performing a primer extension reaction with the first hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension to form a primer extension product;

c) isolating the primer extension product by contacting the primer extension product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated second hybridization product; and

detecting the presence or absence of a label in the isolated second hybridization product, the presence of a label indicating the incorporation of the labeled nucleotide into the primer extension product, and the identity of the identified incorporated labeled nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide in the target nucleic acid.

In a typical assay, the target oligonucleotide is designed such that the 5' end comprises second complementarity region and later allows for hybridization to a complementary capture oligonucleotide linked to a mobile solid support, and the 3' end comprises a first complementarity region complementary a region of the target nucleic acid just 3' of the polymorphic base. The 3' base ends at a nucleotide chosen relative to the polymorphic base, depending upon the assay being performed. For example, the 3' base of this target oligonucleotide can end at the nucleotide 5' to the polymorphic base, or it can end with a base corresponding to the polymorphic base. The present invention additionally provides the use of the beads in an oligonucleotide-ligation assay (OLA) format, i.e., in which one can hybridize genomic DNA, cRNA or PCR product to a target oligonucleotide having a first complementarity region that is complementary to a section of the target nucleic acid that is directly 3' of the



selected nucleotide, then come in with a reporter oligonucleotide having a fluorescent label, then add ligase, and wherein the target oligonucleotide has at its 3' end the polymorphic bases. For a typical OLA reaction with capture read out, the reagents can comprise: a target oligonucleotide containing two regions of complementarity; a  
5 first complementarity region of the target oligo is complementary to a region immediately adjacent to a single nucleotide polymorphism to be analyzed, a second complementarity region of the target oligonucleotide which is complementary to a capture oligonucleotide; a capture oligonucleotide that is covalently coupled to a mobile solid support; a reporter oligonucleotide complementary to the the region  
10 overlapping the SNP and containing a means for readout, and a 3' base on the strand opposite the SNP position; a ligase capable of ligating the reporter and the target if the base on the reporter that is opposite the SNP is complementary. In one embodiment of the method, the ligation reaction is then added to the capture-oligonucleotide-coupled mobile solid support and hybridization of the second complementarity region  
15 to the bead is allowed to occur under standard hybridization conditions. Readout of the reporter could be performed using a Luminex LX100-type machine.

The advantages to this system include the reduced number of bead sets needed to analyze many different SNPs, i.e., if given 100 bead colors, then one could synthesize only 100 capture oligonucleotides and use them over and over again in the  
20 different wells.

Thus, the present invention provides a method of detecting a result from an identification reaction (OLA) to identify a selected nucleotide in a target nucleic acid comprising:

- 5 a) hybridizing (i) a target oligonucleotide comprising a first complementarity region and a second complementarity region, wherein the first complementarity region comprises a region complementary to a section of the target nucleic acid that is directly 3' of and adjacent to the selected nucleotide and the second complementarity region comprises a nucleic acid sequence complementary to a capture oligonucleotide, and (ii) a fluorescently labeled reporter oligonucleotide comprising a region complementary to a section of the target nucleic acid that is directly 5' of and adjacent to the selected nucleotide, to a sample comprising the target nucleic acid, under hybridization conditions that allow specific hybridization between the first complementarity region of the target oligonucleotide and a region of the target nucleic acid complementary to the first complementarity region of the target oligonucleotide and that also allow specific hybridization between the reporter oligonucleotide and the section of the target nucleic acid complementary to the reporter oligonucleotide, to form a first hybridization product that defines a gap opposite the selected nucleotide;
- 10 b) adding an identified test nucleotide, a polymerase and a ligase, under conditions for polymerization and ligation to form a labeled product;
- 15 c) dissociating the hybridized nucleic acids;
- 20 d) isolating the labeled product by contacting the labeled product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence

complementary to the second complementarity region of the target  
oligonucleotide, under hybridization conditions to form a second hybridization  
product; and

e) detecting the presence or absence of the label in the second

5 hybridization product,

the presence of the label indicating polymerization of the identified test  
nucleotide to the target oligonucleotide and ligation of the labeled reporter  
oligonucleotide to the polymerized target oligonucleotide, and the identity of  
the identified test nucleotide indicating the identity of the nucleotide  
10 complementary to the selected nucleotide, thus identifying the selected  
nucleotide in the target nucleic acid.

The target nucleic acid can be genomic DNA treated to reduce viscosity, an  
oligonucleotide, a 16s ribosomal RNA, a 16S DNA, a PCR product, a DNA fragment,  
a restriction enzyme-generated DNA fragment, size-selected DNA, Bridge-amplified  
15 DNA, an RNA molecule, a cDNA molecule or a crRNA molecule.

The present invention further provides a method of determining a selected  
nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising

a) performing an amplification of the genomic DNA using a first nucleic acid  
primer comprising a region complementary to a section of one strand of  
20 the nucleic acid that is 5' of the selected nucleotide, and a second nucleic  
acid primer complementary to a section of the opposite strand of the  
nucleic acid downstream of the selected nucleotide, under conditions for  
specific amplification of the region of the selected nucleotide between the  
two primers, to form a PCR product;

- b) contacting the PCR product with a target oligonucleotide comprising a first complementarity region and a second complementarity region, wherein the first complementarity region is complementary to a section of one strand of the PCR product that is directly 5' of and adjacent to the selected nucleotide, under hybridization conditions to form a first hybridization product;
- c) performing a primer extension reaction with the first hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension to form a primer extension product;
- d) isolating the primer extension product by contacting the primer extension product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated second hybridization product;
- e) detecting the presence or absence of a label incorporated into the second hybridization product, the presence of a label indicating the incorporation of the labeled chain-terminating nucleotide into the primer extension product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide; and
- f) comparing the identity of the selected nucleotide with a non-polymorphic nucleotide, a different identity of the selected nucleotide from that of the

non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.

- The present invention further provides a method of determining a selected
- 5 nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising
- a) performing an amplification of the genomic DNA using as a primer an oligonucleotide comprising a first region having a T7 RNA polymerase promoter and a second region complementary to a section of one strand of the nucleic acid that is directly 5' of the selected nucleotide, and using T7
  - 10 RNA polymerase to amplify one strand into cRNA and using reverse transcriptase to amplify the second strand complementary to the cRNA strand, under conditions for specific amplification of the region of the nucleotide between the two primers, to form an amplification product;
  - b) contacting the amplification product with a target oligonucleotide
  - 15 comprising a first complementarity region and a second complementarity region, wherein the first complementarity region is complementary to a section of one strand of the PCR product that is directly 5' of and adjacent to the selected nucleotide and wherein the second complementarity region is 5' to the first complementarity region, under hybridization conditions to
  - 20 form a first hybridization product;
  - c) performing a primer extension reaction with the first hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension to form a primer extension product;

- d) isolating the primer extension product by contacting the primer extension product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated second hybridization product;
- e) detecting the presence or absence of a label incorporated into the second hybridization product, the presence of a label indicating the incorporation of the labeled chain-terminating nucleotide into the primer extension product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide; and
- f) comparing the identity of the selected nucleotide with a non-polymorphic nucleotide, a different identity of the selected nucleotide from that of the non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.

The labeled chain –terminating nucleotide can be, for example, a 3'deoynucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a dideoxynucleotide. The amplification product can be in single-stranded form. Furthermore, one can design and synthesize some primers to sit just downstream of the target oligonucleotides.

The present method further provides a method of determining a selected nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising

- a) contacting the genomic DNA with a target oligonucleotide comprising a first complementarity region and a second complementarity region,

wherein the first complementarity region is complementary to a section of one strand of the PCR product that is directly 5' of and adjacent to the selected nucleotide and wherein the second complementarity region is 5' to the first complementarity region, under hybridization conditions for forming a specific first hybridization product;

b) performing a primer extension reaction with the specific first hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension;

c) isolating the primer extension product by contacting the primer extension product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated second hybridization product;

d) detecting the presence or absence of a label incorporated into the second hybridization product, the presence of a label indicating the incorporation of the labeled chain-terminating nucleotide into the hybridization product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide; and

e) comparing the identity of the selected nucleotide with a non-polymorphic nucleotide, a different identity of the selected nucleotide from that of the non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.

The DNA can be in single-stranded form. The labeled chain –terminating nucleotide can be, for example, a 3'deoynucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a dideoxynucleotide. In such a method, the hybridization time should be of a length sufficient to allow hybridization of the first primer to the genomic DNA  
5 since the genomic DNA has not been amplified in this specific embodiment. Thus relatively long hybridization times may be utilized, such as, for example, 12 hours, 24 hours, 48 hours, as is known in the art for hybridization to genomic DNA (*see, e.g.,* Sambrook, *et al.*).

In reactions utilizing a ligase, any selected ligase can be used, such as T4 DNA  
10 ligase. A thermostable ligase would be particularly useful. *See, generally* Wu and Wallace, *Genomics* 4: 560-569 (1989).

The invention further employs hybridization methods wherein two nucleic acids are hybridized to the sample nucleic acid but the step of ligation can be omitted and a match instead detected by fluorescence energy transfer between the two nucleic  
15 acids hybridized to the sample nucleic acid. The two hybridizing nucleic acids are designed such that the 3' end of the target oligonucleotide is a test base, and when it is complementary to the polymorphic base, and a single wavelength of light is directed onto the sample, one can detect a transfer of energy, read as a second wavelength of light. A second reader can be employed for this detection of this second wavelength.  
20 Thus, the present invention provides a method of identifying a selected nucleotide in a target nucleic acid comprising

- a) contacting the target nucleic acid with
  - i. a target oligonucleotide comprising a first complementarity region and a second complementarity region, wherein the first complementarity



- region is complementary to a section of the first nucleic acid that is directly 5' of the selected nucleotide, wherein the target oligonucleotide terminates at its 3' end in an identified test nucleotide positioned to base-pair with the selected nucleotide, and wherein the second
- 5        complementarity region is 5' to the first complementarity region, and
- ii. a fluorescently labeled reporter oligonucleotide, wherein the reporter oligonucleotide comprises a region complementary to a section of the target nucleic acid that is directly 3' of and adjacent to the selected nucleotide,
- 10        under hybridization conditions that allow the target nucleic acid and the target oligonucleotide to hybridize and the target nucleic acid and the reporter oligonucleotide to hybridize, thus forming a first hybridization product;
- b) adding to the first hybridization product a ligase under ligation conditions;
- 15        c) isolating the first hybridization product by contacting the first hybridization product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization
- 20        conditions to form an isolated second hybridization product; and
- detecting the presence or absence of the fluorescent label, after dissociation of the hybridized nucleic acids, in the second hybridization product, the presence of the label indicating the ligation of the labeled reporter oligonucleotide to the target oligonucleotide, and the identity of the test nucleotide in the target oligonucleotide

indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide. The detection of the fluorescence energy transfer can be performed after dissociation of the hybridized nucleic acids.

The present invention additionally provides a method of identifying a selected  
5 nucleotide in a target nucleic acid comprising

- a) contacting the target nucleic acid with
  - i. a target oligonucleotide linked at its 3' end to a fluorescent label, wherein the target oligonucleotide comprises a first complementarity region that is complementary to a section of the target nucleic acid that is directly 3' of  
10 the selected nucleotide, wherein the target oligonucleotide terminates at its 3' end in a test nucleotide positioned to base-pair with the selected nucleotide, and wherein the target oligonucleotide has a second complementarity region 5' of the first complementarity region, and
  - ii. a reporter oligonucleotide fluorescently labeled at its 5' end, wherein the  
15 reporter oligonucleotide comprises a region complementary to a section of the target nucleic acid that is directly 5' of and adjacent to the selected nucleotide,

under hybridization conditions that allow the target nucleic acid and the target  
oligonucleotide to hybridize and the target nucleic acid and the reporter  
20 oligonucleotide to hybridize, to form a first hybridization product;

- b) isolating the first hybridization product by contacting the first hybridization product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target

oligonucleotide, under hybridization conditions to form an isolated second hybridization product; and

detecting the presence or absence of fluorescent energy transfer between the fluorescent label at the 3' end of the target oligonucleotide and the fluorescent label at the 5' end of the reporter oligonucleotide in the second hybridization product, the presence of fluorescent energy transfer indicating the hybridization of the identified test nucleotide to the target nucleic acid, and the identity of the hybridized test nucleotide in the target oligonucleotide indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide.

10 The present invention also provides a method for determining the sequence of a polymorphic base in a target nucleic acid which can utilize a kit comprising one or more of the following: a target oligonucleotide, wherein the target oligonucleotide comprises a first complementarity region and a second complementarity region 5' of the first complementarity region, wherein the first complementarity region is  
15 complementary to a section of the target nucleic acid having a 3' end adjacent to and directly 5' of the polymorphic base on the target nucleic acid; a reporter oligonucleotide with an attached reporter moiety that is complementary to a region immediately adjacent to and 3' of the polymorphic base of the target nucleic acid; the target oligonucleotide and the reporter oligonucleotide together defining a gap  
20 opposite the polymorphic base; a capture oligonucleotide that is covalently linked to a mobile solid support (such as a polystyrene-divinylbenzene bead), wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide; a nucleotide that is complementary to one of a set of two possible polymorphic bases; a polymerase, and

a ligase, wherein the polymerase is able to polymerize the nucleotide across the gap if the nucleotide is complementary to the polymorphic base and wherein the ligase is able to ligate the newly polymerized nucleotide to the reporter oligonucleotide; and a means for detecting the reporter covalently linked to the bead. Further, the present invention provides a method of identifying a selected nucleotide in a target nucleic acid comprising

- a) contacting the target nucleic acid with
  - i. a target oligonucleotide, wherein the target oligonucleotide comprises a first complementarity region and a second complementarity region 5' of the first complementarity region, wherein the first complementarity region is complementary to a section of the target nucleic acid that is directly 3' of and immediately adjacent to the selected nucleotide, and
  - ii. a reporter oligonucleotide fluorescently labeled, wherein the reporter oligonucleotide comprises a region complementary to a section of the second nucleic acid that is directly 5' of and adjacent to the selected nucleotide,

under hybridization conditions that allow the target nucleic acid and the target oligonucleotide to form a hybridization product and the target nucleic acid and the reporter oligonucleotide to form a hybridization product, wherein the target nucleic acid, target oligonucleotide and reporter oligonucleotide form a hybridization product that defines a gap opposite the selected nucleotide;

- b) adding an identified test nucleotide, a polymerase and a ligase, under conditions for polymerization and ligation;

- c) isolating the first hybridization product by contacting the first hybridization product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target  
5 oligonucleotide, under hybridization conditions to form an isolated second hybridization product; and
- d) detecting the presence or absence of the fluorescent label, after dissociation of the hybridized nucleic acids, in the second hybridization product, the presence of the label indicating the polymerization of the test nucleic acid to the  
10 target oligonucleotide and ligation of the labeled reporter oligonucleotide to the target oligonucleotide linked to the mobile solid support, and the identity of the test nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide.

The polymerase can preferably be a non-strand displacing polymerase.  
15 Further, it can be a thermostable polymerase. The ligase can be a DNA ligase. Further, it can be a thermostable ligase.

Furthermore, any of the herein described methods can be utilized in a method for quantitating expression of a selected nucleic acid in a sample. Thus, it can be used, for example, for differential gene expression wherein the expression of a  
20 selected gene is quantitated and compared to a standard or some other reference. For this method, a gene fragment from a region of interest or a region that distinguishes the gene (or allele or haplotype or polymorphism) of interest is selected for use as the first complementarity region of a target oligonucleotide; message (e.g., RNA, cDNA, cRNA) is hybridized to the target oligonucleotide, and fluorescence is quantitated by

performing a primer extension reaction, a ligase reaction or a hybridization/fluorescence energy transfer reaction, such as that described herein. A corresponding capture oligonucleotide (complementary to a second complementarity region utilized in the target oligonucleotide) linked to a mobile solid support is  
5 utilized to capture the reaction product. The first complementarity region of a target oligonucleotide can comprise a region complementary to a section of the selected nucleic acid unique to the nucleic acid. A standard, such as that from a normal subject, or a diseased/ afflicted subject, or a particular tissue or organ, or a particular species, can be used as a comparison reference to draw conclusions regarding the  
10 quantity detected in the sample.

Thus the present invention provides a method of quantitating expression of a selected nucleic acid in a sample comprising

- a) contacting (i) message nucleic acid isolated from a selected source with (ii) a target oligonucleotide, wherein the target oligonucleotide comprises a first  
15 complementarity region and a second complementarity region 5' of the first complementarity region, wherein the first complementarity region comprises a region complementary to a section of the selected nucleic acid;
- b) performing a selected identification reaction with the first hybridization product to determine the identity of the selected nucleotide wherein a  
20 selectively labeled detection product comprising the second complementarity region of the target oligonucleotide can be formed;
- c) isolating the detection product by contacting the detection product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence

complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated hybridization product; and

- d) quantitating the fluorescence in the isolated hybridization product, the quantity of fluorescence indicating the quantity of the selected nucleic acid in the sample.

For any of these methods described herein, a sample can be, for example, any body sample that contains message, such as organ tissue and/or cells, such as blood, red or white blood cells, bone marrow, liver, kidney, brain, skin, heart, lung, spleen, pancreas, gall bladder, muscle, neuronal cells, neurons, precursor cells, ovaries, testicles, uterus, glands.

Additionally provided are kits for detecting a single base polymorphism, wherein a kit comprises a detectably tagged mobile solid support, such as a polystyrene-divinylbenzene bead, and one to four modified (chain-terminating) nucleotide(s), such as a 3' deoxynucleotide, a 3' deoxyribonucleotide, a thiol derivative, or a dideoxynucleotide. The kit can additionally comprise a polymerase, and in particular, a polymerase that preferentially incorporates the modified nucleotide. The kit can additionally comprise a ligase. The kit can also comprise one or more fluorescent label for labeling the nucleic acid(s). For genomic DNA uses, the kit can further comprises a DNase for reducing the viscosity of the DNA. The kit can further contain an array of combinations of dinucleotides and/or a collection of combinations of trinucleotides.

The following documents provide information regarding various technologies:

PCT publication WO 9714028 (Luminex Corp.).

Australian patent AU 9723205 (based on WO 9735033 (97/09/25)) (Molecular Tool Inc.)

European patent publication EP 754240 (based on WO 9521271) (Molecular Tool Inc.)

European patent publication EP 736107 (based on WO 9517524) (Molecular Tool Inc.)

U.S. Pat. No. 5,610,287 (97/03/11) (Molecular Tool Inc.)

European patent publication EP 726905 (based on WO 9512607) (Molecular Tool Inc.)

U.S. Pat. No. 5,518,900 (94/07/21) (Molecular Tool Inc.)

European patent publication EP 576558 (based on WO 9215712) (Molecular Tool Inc.)

Throughout this application, various publication are referenced. These publications are hereby incorporated by reference in their entirety.

While the invention has been described with respect to certain specific embodiments, it will be appreciated that many modifications and changes may be made by those skilled in the art without departing from the spirit of the invention. It is intended, therefore, by the appended claims, to cover all such modification and changes as fall within the true spirit and scope of the invention.



**What is claimed is:**

1. A method of identifying a selected nucleotide in a first nucleic acid comprising
  - a) contacting the first nucleic acid with a nucleic acid primer linked at its 5'  
5 end to a detectably tagged mobile solid support wherein the nucleic acid primer comprises a region complementary to a section of the first nucleic acid that is directly 3' of and adjacent to the selected nucleotide, under hybridization conditions that allow the first nucleic acid and the nucleic acid primer to form a hybridization product;
  - 10 b) performing a primer extension reaction with the hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension; and
  - c) detecting the presence or absence of a label incorporated into the hybridization product, the presence of a label indicating the incorporation  
15 of the labeled nucleotide into the hybridization product, and the identity of the incorporated labeled nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide in the first nucleic acid.
- 20 2. The method of claim 1, wherein the first nucleic acid is an oligonucleotide, a 16s ribosomal RNA, a PCR product, a DNA fragment, an RNA molecule, a cDNA molecule or a cRNA molecule, the nucleic acid primer is an oligonucleotide, a PCR product, a DNA fragment, an RNA molecule, a cDNA molecule or a cRNA molecule.

3. The method of claim 1, wherein the labeled chain –terminating nucleotide is a 3'deoxy nucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a dideoxynucleotide.
4. The method of claim 1, wherein the chain terminating nucleotide is a dideoxynucleotide and the primer extension is performed in the presence of one labeled, identified dideoxynucleotide and three different, non-labeled dideoxynucleotides.
5. The method of claim 1, wherein the chain terminating nucleotide is a dideoxynucleotide, wherein the primer extension is performed in the presence of a first identified dideoxynucleotide labeled with a first detectable label, a second identified dideoxynucleotide labeled with a second detectable label, a third identified dideoxynucleotide labeled with a third detectable label and a fourth identified dideoxynucleotide labeled with a fourth detectable label, and wherein detection of the presence of the first, the second, the third or the fourth detectable label in the hybridization product indicates the identity of the nucleotide complementary to the selected nucleotide as the first, the second, the third or the fourth dideoxynucleotide, respectively.
6. The method of claim 1, wherein the mobile solid support is a bead.
7. The method of claim 4, wherein the bead is a polystyrene-divinylbenzene.
8. The method of claim 1, wherein the mobile solid support is detectably tagged with dye, a radiolabel, or a magnetic tag.
9. The method of claim 1, wherein the first nucleic acid is an amplification product.
10. The method of claim 1, wherein the first nucleic acid is a PCR product.

11. The method of claim 1, wherein the detecting is performed by passing the mobile solid support over a laser detection device capable of detecting/distinguishing the detectable tag.
12. The method of claim 1, wherein the detecting is performed by fixing the mobile solid support onto a two-dimensional surface and passing a laser detection device capable of detecting/distinguishing the detectable tag over the solid support.
13. A method of determining a selected nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising
- a) performing an amplification of the genomic DNA using a first nucleic acid primer comprising a region complementary to a section of one strand of the nucleic acid that is 5' of the selected nucleotide, and a second nucleic acid primer complimentary to a section of the opposite strand of the nucleic acid downstream of the selected nucleotide, under conditions for specific amplification of the region of the selected nucleotide between the two primers, to form a PCR product;
  - b) contacting the PCR product with a first nucleic acid linked at its 5' end to a detectably tagged mobile solid support, wherein the first nucleic acid comprises a region complementary to a section of one strand of the PCR product that is directly 5' of and adjacent to the selected nucleotide, under hybridization conditions to form a hybridization product;
  - c) performing a primer extension reaction with the hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension;

- d) detecting the presence or absence of a label incorporated into the hybridization product, the presence of a label indicating the incorporation of the labeled chain-terminating nucleotide into the hybridization product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide; and
- e) comparing the identity of the selected nucleotide with a non-polymorphic nucleotide, a different identity of the selected nucleotide from that of the non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.
14. The method of claim 13, wherein the labeled chain-terminating nucleotide is a 3'deoxynucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a dideoxynucleotide.
15. The method of claim 13, wherein the DNA has been sheared to reduce viscosity.
16. The method of claim 13, wherein the DNA has been treated with DNase to reduce viscosity.
17. A method of determining a selected nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising
- a) performing an amplification of the genomic DNA using as a primer an oligonucleotide comprising a first region having a T7 RNA polymerase promoter and a second region complementary to a section of one strand of the nucleic acid that is directly 5' of the selected nucleotide, and using T7 RNA polymerase to amplify one strand into cRNA and using reverse transcriptase to amplify the second strand complementary to the cRNA

- strand, under conditions for specific amplification of the region of the nucleotide between the two primers, to form an amplification product;
- b) contacting the amplification product with a first oligonucleotide linked at its 5' end to a detectably tagged mobile solid support under hybridization conditions to form a hybridization product;
- 5 c) performing a primer extension reaction with the hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension;
- d) detecting the presence or absence of a label incorporated into the hybridization product, the presence of a label indicating the incorporation of the labeled chain-terminating nucleotide into the hybridization product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide; and
- 10 e) comparing the identity of the selected nucleotide with a non-polymorphic nucleotide, a different identity of the selected nucleotide from that of the non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.
- 15

18. The method of claim 17, wherein the labeled chain –terminating nucleotide is a 3'deoynucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a dideoxynucleotide.

20

19. A method of determining a selected nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising

- 5 a) contacting the genomic DNA with a first primer linked at its 5' end to a detectably tagged mobile solid support, wherein the first primer comprises a first region complementary to a section of one strand of the genomic DNA that is directly 5' of and adjacent to the selected nucleotide under hybridization conditions for forming a specific hybridization product;
- b) performing a primer extension reaction with the specific hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension;
- 10 c) detecting the presence or absence of a label incorporated into the hybridization product, the presence of a label indicating the incorporation of the labeled chain-terminating nucleotide into the hybridization product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide; and
- 15 d) comparing the identity of the selected nucleotide with a non-polymorphic nucleotide, a different identity of the selected nucleotide from that of the non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.

20. The method of claim 19, wherein the labeled chain –terminating nucleotide is a 3'deoynucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a dideoxynucleotide.

21. A method of identifying a selected nucleotide in a first nucleic acid comprising

- a) contacting the first nucleic acid with

- 5 i. a second nucleic acid linked at its 5' end to a detectably tagged mobile solid support wherein the second nucleic acid comprises a region complementary to a section of the first nucleic acid that is directly 3' of the selected nucleotide and wherein the second nucleic acid terminates at its 3' end in a test nucleotide positioned to base-pair with the selected nucleotide, and
- 10 ii. a third, fluorescently labeled nucleic acid, wherein the third nucleic acid comprises a region complementary to a section of the second nucleic acid that is directly 5' of and adjacent to the selected nucleotide,
- under hybridization conditions that allow the first nucleic acid and the second nucleic acid to form a hybridization product and the first nucleic acid and the third nucleic acid to form a hybridization product;
- 15 b) adding to the hybridization product a ligase under ligation conditions; and
- c) detecting the presence or absence of the fluorescent label, after dissociation of the hybridized nucleic acids, in the nucleic acid linked to the mobile solid support, the presence of the label indicating the ligation of the labeled third nucleic acid to the second nucleic acid linked to the mobile solid support, and the identity of the test nucleotide in the second
- 20 nucleic acid indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide.

22. The method of claim 21, wherein the first nucleic acid is genomic DNA treated to reduce viscosity, an oligonucleotide, a 16s ribosomal RNA, a PCR product, a DNA fragment, an RNA molecule, a cDNA molecule or a rRNA molecule.

23. A method of identifying a selected nucleotide in a first nucleic acid comprising

a) contacting the first nucleic acid with

i. a second nucleic acid linked at its 5' end to a detectably tagged mobile solid

support and linked at its 3' end to a fluorescent label, wherein the

5 second nucleic acid comprises a region complementary to a section of

the first nucleic acid that is directly 3' of the selected nucleotide and

wherein the second nucleic acid terminates at its 3' end in a test

nucleotide positioned to base-pair with the selected nucleotide, and

ii. a third nucleic acid fluorescently labeled at its 5' end, wherein the third

10 nucleic acid comprises a region complementary to a section of the

second nucleic acid that is directly 5' of and adjacent to the selected

nucleotide,

under hybridization conditions that allow the first nucleic acid and the second

nucleic acid to form a hybridization product and the first nucleic acid and the third

15 nucleic acid to form a hybridization product; and

b) detecting the presence or absence of fluorescent energy transfer between the

fluorescent label at the 3' end of the second nucleic acid and the fluorescent

label at the 5' end of the third nucleic acid, the presence of fluorescent energy

transfer indicating the hybridization of the test nucleotide to the first nucleic

20 acid, and the identity of the hybridized test nucleotide in the second nucleic

acid indicating the identity of the nucleotide complementary to the selected

nucleotide, thus identifying the selected nucleotide.

24. A method of identifying a selected nucleotide in a first nucleic acid comprising

a) contacting the first nucleic acid with



- i. a second nucleic acid linked at its 5' end to a detectably tagged mobile solid support, wherein the second nucleic acid comprises a region complementary to a section of the first nucleic acid that is directly 3' of and immediately adjacent to the selected nucleotide, and
- 5 ii. a third nucleic acid fluorescently labeled, wherein the third nucleic acid comprises a region complementary to a section of the second nucleic acid that is directly 5' of and adjacent to the selected nucleotide,  
under hybridization conditions that allow the first nucleic acid and the second nucleic acid to form a hybridization product and the first nucleic acid and the third  
10 nucleic acid to form a hybridization product, wherein the first, second and third nucleic acids form a hybridization product that defines a gap opposite the selected nucleotide;
  - b) adding a test nucleotide, a polymerase and a ligase, under conditions for polymerization and ligation; and
  - 15 c) detecting the presence or absence of the fluorescent label, after dissociation of the hybridized nucleic acids, in the nucleic acid linked to the mobile solid support,  
the presence of the label indicating the polymerization of the test nucleic acid to the second nucleic acid and ligation of the labeled third nucleic acid to the second nucleic  
20 acid linked to the mobile solid support, and the identity of the test nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide.
25. A method of quantitating expression of a selected nucleic acid in a sample comprising

- a) contacting (i) message nucleic acid isolated from a selected source with (ii) a nucleic acid probe linked at its 5' end to a detectably tagged mobile solid support, wherein the nucleic acid probe comprises a region complementary to a section of the selected nucleic acid;
- 5 b) performing the detection method of any of claims 1, 13, 17, 19, 21, 22, 23, and 24; and
- c) quantitating the fluorescence linked to the mobile solid support, the quantity of fluorescence indicating the quantity of the selected nucleic acid in the sample.

26. The method of claim 25, wherein the message nucleic acid is mRNA, cRNA or  
10 cDNA.

27. A method of detecting a result from an identification reaction to identify a selected nucleotide in a target nucleic acid comprising:
- a) contacting a target oligonucleotide comprising a first complementarity  
15 region and a second complementarity region, wherein the second complementarity region is 5' of the first complementarity region and wherein the first complementarity region comprises a region complementary to a section of the target nucleic acid that is directly 3' of and adjacent to the selected nucleotide, with a sample comprising the  
20 target nucleic acid, under hybridization conditions that allow the formation of a hybridization product between the first complementarity region of the target oligonucleotide and a region of the target nucleic acid complementary to the first complementarity region of the target oligonucleotide, to form a first hybridization product;

- b) performing a selected identification reaction with the first hybridization product to determine the identity of the selected nucleotide wherein a selectively labeled detection product comprising the second complementarity region of the target oligonucleotide can be formed;
- 5 c) isolating the detection product by contacting the detection product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form a second
- 10 hybridization product; and
- d) detecting and/or identifying the label of the labeled detection product in the second hybridization product,
- the presence and or identity of the label indicating the identity of the selected nucleotide in the target nucleic acid.
- 15 28. The method of claim 27, wherein the identification reaction is an oligonucleotide ligation reaction.
29. The method of claim 27, wherein the identification reaction comprises performing a primer extension reaction with the first hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer
- 20 extension; and wherein the detection comprises detecting the presence or absence of a label incorporated into the second hybridization product, the presence of a label indicating the incorporation of the labeled nucleotide into the first hybridization product, and the identity of the incorporated labeled nucleotide

indicating the identity of the nucleotide complementary to the selected nucleotide,  
thus identifying the selected nucleotide in the target nucleic acid.

30. The method of claim 27, wherein the target nucleic acid is an oligonucleotide, a  
16s ribosomal RNA, a PCR product, a DNA fragment, an RNA molecule, a  
5 cDNA molecule or a cRNA molecule, the nucleic acid primer is an  
oligonucleotide, a PCR product, a DNA fragment, an RNA molecule, a cDNA  
molecule or a cRNA molecule.
31. The method of claim 27, wherein the mobile solid support is a bead.
32. The method of claim 31, wherein the bead is a polystyrene-divinylbenzene.
- 10 33. The method of claim 27, wherein the mobile solid support is detectably tagged  
with dye, a radiolabel, or a magnetic tag.
34. The method of claim 27, wherein the first nucleic acid is an amplification product.
35. The method of claim 27, wherein the first nucleic acid is a PCR product.
36. The method of claim 27, wherein the detecting is performed by passing the mobile  
15 solid support over a laser detection device capable of detecting/distinguishing the  
detectable tag.
37. The method of claim 27, wherein the detecting is performed by fixing the mobile  
solid support onto a two-dimensional surface and passing a laser detection device  
capable of detecting/distinguishing the detectable tag over the solid support.
- 20 38. A method of detecting a reaction product to identify a selected nucleotide in a  
target nucleic acid comprising:
- a) contacting a target oligonucleotide comprising a first complementarity  
region and a second complementarity region, wherein the first  
complementarity region comprises the oligonucleotide primer and the

- second complementarity region comprises a nucleic acid sequence complementary to a capture oligonucleotide, and wherein the oligonucleotide primer comprises a region complementary to a section of the target nucleic acid that is directly 3' of and adjacent to the selected nucleotide, with a sample comprising the target nucleic acid, under hybridization conditions that allow the formation of a hybridization product between the first complementarity region of the target oligonucleotide and a region of the target nucleic acid complementary to the first complementarity region of the target oligonucleotide, to form a first hybridization product;
- b) performing a primer extension reaction with the first hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension to form a primer extension product;
- c) isolating the primer extension product by contacting the primer extension product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated second hybridization product; and
- d) detecting the presence or absence of a label in the isolated second hybridization product, the presence of a label indicating the incorporation of the labeled nucleotide into the primer extension product, and the identity of the identified incorporated labeled nucleotide indicating the

identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide in the target nucleic acid.

39. The method of claim 38, wherein the target nucleic acid is an oligonucleotide, a 16s ribosomal RNA, a PCR product, a DNA fragment, an RNA molecule, a cDNA molecule or a cRNA molecule, the nucleic acid primer is an oligonucleotide, a PCR product, a DNA fragment, an RNA molecule, a cDNA molecule or a cRNA molecule.
40. The method of claim 39, wherein the labeled chain-terminating nucleotide is a 3'deoynucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a dideoxynucleotide.
41. The method of claim 39, wherein the chain terminating nucleotide is a dideoxynucleotide and the primer extension is performed in the presence of one labeled, identified dideoxynucleotide and three different, non-labeled dideoxynucleotides.
42. The method of claim 39, wherein the chain terminating nucleotide is a dideoxynucleotide, wherein the primer extension is performed in the presence of a first identified dideoxynucleotide labeled with a first detectable label, a second identified dideoxynucleotide labeled with a second detectable label, a third identified dideoxynucleotide labeled with a third detectable label and a fourth identified dideoxynucleotide labeled with a fourth detectable label, and wherein detection of the presence of the first, the second, the third or the fourth detectable label in the second hybridization product indicates the identity of the nucleotide complementary to the selected nucleotide as the first, the second, the third or the fourth dideoxynucleotide, respectively.

43. The method of claim 39, wherein the mobile solid support is a bead.
44. The method of claim 43, wherein the bead is a polystyrene-divinylbenzene.
45. The method of claim 43, wherein the mobile solid support is detectably tagged with dye, a radiolabel, or a magnetic tag.
- 5 46. The method of claim 39, wherein the target nucleic acid is an amplification product.
47. The method of claim 39, wherein the target nucleic acid is a PCR product.
48. The method of claim 39, wherein the detecting is performed by passing the mobile solid support over a laser detection device capable of detecting/distinguishing the  
10 detectable label.
49. The method of claim 39, wherein the detecting is performed by fixing the mobile solid support onto a two-dimensional surface and passing a laser detection device capable of detecting/distinguishing the detectable label over the solid support.
50. A method of detecting a result from an identification reaction to identify a  
15 selected nucleotide in a target nucleic acid comprising:
- a) hybridizing (i) a target oligonucleotide comprising a first complementarity region and a second complementarity region, wherein the first complementarity region comprises a region complementary to a section of the target nucleic acid that is directly 3' of and adjacent to the selected  
20 nucleotide and the second complementarity region comprises a nucleic acid sequence complementary to a capture oligonucleotide, and (ii) a fluorescently labeled reporter oligonucleotide comprising a region complementary to a section of the target nucleic acid that is directly 5' of and adjacent to the selected nucleotide, to a sample comprising the target

- nucleic acid, under hybridization conditions that allow specific hybridization between the first complementarity region of the target oligonucleotide and a region of the target nucleic acid complementary to the first complementarity region of the target oligonucleotide and that also allow specific hybridization between the reporter oligonucleotide and the section of the target nucleic acid complementary to the reporter oligonucleotide, to form a first hybridization product that defines a gap opposite the selected nucleotide;
- b) adding an identified test nucleotide, a polymerase and a ligase, under conditions for polymerization and ligation to form a labeled product;
- c) dissociating the hybridized nucleic acids;
- d) isolating the labeled product by contacting the labeled product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form a second hybridization product; and
- e) detecting the presence or absence of the label in the second hybridization product,
- the presence of the label indicating polymerization of the identified test nucleotide to the target oligonucleotide and ligation of the labeled reporter oligonucleotide to the polymerized target oligonucleotide, and the identity of the identified test nucleotide indicating the identity of the nucleotide



complementary to the selected nucleotide, thus identifying the selected nucleotide in the target nucleic acid.

51. The method of claim 50, wherein the target nucleic acid is an oligonucleotide, a 16s ribosomal RNA, a PCR product, a DNA fragment, an RNA molecule, a cDNA molecule or a cRNA molecule, the nucleic acid primer is an oligonucleotide, a PCR product, a DNA fragment, an RNA molecule, a cDNA molecule or a cRNA molecule.
52. The method of claim 50, wherein the reporter oligonucleotide is selected from the group consisting of the 1024 member set of all possible 5-mer oligonucleotides.
- 10 53. The method of claim 50, wherein the mobile solid support is a bead.
54. The method of claim 53, wherein the bead is a polystyrene-divinylbenzene.
55. The method of claim 50, wherein the mobile solid support is detectably tagged with dye, a radiolabel, or a magnetic tag.
56. The method of claim 50, wherein the target nucleic acid is an amplification product.
- 15 57. The method of claim 50, wherein the target nucleic acid is a PCR product.
58. The method of claim 50, wherein the detecting is performed by passing the mobile solid support over a laser detection device capable of detecting/distinguishing the detectable label.
- 20 59. The method of claim 50, wherein the detecting is performed by fixing the mobile solid support onto a two-dimensional surface and passing a laser detection device capable of detecting/distinguishing the detectable label over the solid support.
60. A method of determining a selected nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising

- 5 a) performing an amplification of the genomic DNA using a first nucleic acid primer comprising a region complementary to a section of one strand of the nucleic acid that is 5' of the selected nucleotide, and a second nucleic acid primer complementary to a section of the opposite strand of the nucleic acid downstream of the selected nucleotide, under conditions for specific amplification of the region of the selected nucleotide between the two primers, to form a PCR product;
- 10 b) contacting the PCR product with a target oligonucleotide comprising a first complementarity region and a second complementarity region, wherein the first complementarity region is complementary to a section of one strand of the PCR product that is directly 5' of and adjacent to the selected nucleotide, under hybridization conditions to form a first hybridization product;
- 15 c) performing a primer extension reaction with the first hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension to form a primer extension product;
- 20 d) isolating the primer extension product by contacting the primer extension product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated second hybridization product;
- e) detecting the presence or absence of a label incorporated into the second hybridization product, the presence of a label indicating the incorporation

of the labeled chain-terminating nucleotide into the primer extension product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide; and

- 5           f) comparing the identity of the selected nucleotide with a non-polymorphic nucleotide, a different identity of the selected nucleotide from that of the non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.

61. The method of claim 60, wherein the labeled chain –terminating nucleotide is a  
10       3'deoynucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a dideoxynucleotide.

62. The method of claim 60, wherein the DNA has been sheared to reduce viscosity.

63. The method of claim 60, wherein the DNA has been treated with DNase to reduce viscosity.

15       64. A method of determining a selected nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising

- a) performing an amplification of the genomic DNA using as a primer an oligonucleotide comprising a first region having a T7 RNA polymerase promoter and a second region complementary to a section of one strand of  
20       the nucleic acid that is directly 5' of the selected nucleotide, and using T7 RNA polymerase to amplify one strand into cRNA and using reverse transcriptase to amplify the second strand complementary to the cRNA strand, under conditions for specific amplification of the region of the nucleotide between the two primers, to form an amplification product;

- b) contacting the amplification product with a target oligonucleotide comprising a first complementarity region and a second complementarity region, wherein the first complementarity region is complementary to a section of one strand of the PCR product that is directly 5' of and adjacent to the selected nucleotide and wherein the second complementarity region is 5' to the first complementarity region, under hybridization conditions to form a first hybridization product;
- c) performing a primer extension reaction with the first hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension to form a primer extension product;
- d) isolating the primer extension product by contacting the primer extension product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated second hybridization product;
- e) detecting the presence or absence of a label incorporated into the second hybridization product, the presence of a label indicating the incorporation of the labeled chain-terminating nucleotide into the primer extension product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide; and
- f) comparing the identity of the selected nucleotide with a non-polymorphic nucleotide, a different identity of the selected nucleotide from that of the

non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.

65. The method of claim 64, wherein the labeled chain –terminating nucleotide is a 3'deoynucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a dideoxynucleotide.

66. A method of determining a selected nucleotide polymorphism in genomic DNA treated to reduce viscosity comprising

a) contacting the genomic DNA with a target oligonucleotide comprising a first complementarity region and a second complementarity region,

10 wherein the first complementarity region is complementary to a section of one strand of the PCR product that is directly 5' of and adjacent to the selected nucleotide and wherein the second complementarity region is 5' to the first complementarity region, under hybridization conditions for forming a specific first hybridization product;

15 b) performing a primer extension reaction with the specific first hybridization product and a detectably labeled, identified chain-terminating nucleotide under conditions for primer extension;

c) isolating the primer extension product by contacting the primer extension product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated second hybridization product;

20

- 5 d) detecting the presence or absence of a label incorporated into the second hybridization product, the presence of a label indicating the incorporation of the labeled chain-terminating nucleotide into the hybridization product, and the identity of the incorporated labeled chain-terminating nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide; and
- 10 e) comparing the identity of the selected nucleotide with a non-polymorphic nucleotide, a different identity of the selected nucleotide from that of the non-polymorphic nucleotide indicating a polymorphism of that selected nucleotide.

67. The method of claim 66, wherein the labeled chain –terminating nucleotide is a 3'deoxy nucleotide, a 3'deoxyribonucleotide, a thiol nucleotide derivative or a dideoxy nucleotide.

68. A method of identifying a selected nucleotide in a target nucleic acid comprising

- 15 a) contacting the target nucleic acid with
- 20 i. a target oligonucleotide comprising a first complementarity region and a second complementarity region, wherein the first complementarity region is complementary to a section of the first nucleic acid that is directly 5' of the selected nucleotide, wherein the target oligonucleotide terminates at its 3' end in an identified test nucleotide positioned to base-pair with the selected nucleotide, and wherein the second complementarity region is 5' to the first complementarity region, and

- ii. a fluorescently labeled reporter oligonucleotide, wherein the reporter oligonucleotide comprises a region complementary to a section of the target nucleic acid that is directly 3' of and adjacent to the selected nucleotide,

5 under hybridization conditions that allow the target nucleic acid and the target oligonucleotide to hybridize and the target nucleic acid and the reporter oligonucleotide to hybridize, thus forming a first hybridization product;

- b) adding to the first hybridization product a ligase under ligation conditions;

10 c) isolating the first hybridization product by contacting the first hybridization product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated second hybridization product; and

15 d) detecting the presence or absence of the fluorescent label, after dissociation of the hybridized nucleic acids, in the second hybridization product, the presence of the label indicating the ligation of the labeled reporter oligonucleotide to the target oligonucleotide, and the identity of the test nucleotide in the target oligonucleotide indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying  
20 the selected nucleotide.

69. The method of claim 68, wherein the first nucleic acid is genomic DNA treated to reduce viscosity, an oligonucleotide, a 16s ribosomal RNA, a PCR product, a DNA fragment, an RNA molecule, a cDNA molecule or a crRNA molecule.

70. A method of identifying a selected nucleotide in a target nucleic acid comprising
- a) contacting the target nucleic acid with
    - i. a target oligonucleotide linked at its 3' end to a fluorescent label, wherein the target oligonucleotide comprises a first complementarity region that is  
5 complementary to a section of the target nucleic acid that is directly 3' of the selected nucleotide, wherein the target oligonucleotide terminates at its 3' end in a test nucleotide positioned to base-pair with the selected nucleotide, and wherein the target oligonucleotide has a second complementarity region 5' of the first complementarity region, and  
10 ii. a reporter oligonucleotide fluorescently labeled at its 5' end, wherein the reporter oligonucleotide comprises a region complementary to a section of the target nucleic acid that is directly 5' of and adjacent to the selected nucleotide, under hybridization conditions that allow the target nucleic acid and the target oligonucleotide to hybridize and the target nucleic acid and the reporter  
15 oligonucleotide to hybridize, to form a first hybridization product;
  - b) isolating the first hybridization product by contacting the first hybridization product with a capture oligonucleotide that is covalently coupled to a mobile solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide,  
20 under hybridization conditions to form an isolated second hybridization product; and
  - d) detecting the presence or absence of fluorescent energy transfer between the fluorescent label at the 3' end of the target oligonucleotide and the fluorescent label at the 5' end of the reporter oligonucleotide in the second hybridization product, the presence of fluorescent energy transfer indicating the hybridization of



the identified test nucleotide to the target nucleic acid, and the identity of the hybridized test nucleotide in the target oligonucleotide indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide.

5 71. A method of identifying a selected nucleotide in a target nucleic acid comprising

a) contacting the target nucleic acid with

- 10 i. a target oligonucleotide, wherein the target oligonucleotide comprises a first complementarity region and a second complementarity region 5' of the first complementarity region, wherein the first complementarity region is complementary to a section of the target nucleic acid that is directly 3' of and immediately adjacent to the selected nucleotide, and
- ii. a reporter oligonucleotide fluorescently labeled, wherein the reporter oligonucleotide comprises a region complementary to a section of the second nucleic acid that is directly 5' of and adjacent to the selected
- 15 nucleotide,

under hybridization conditions that allow the target nucleic acid and the target oligonucleotide to form a hybridization product and the target nucleic acid and the reporter oligonucleotide to form a hybridization product, wherein the target nucleic acid, target oligonucleotide and reporter oligonucleotide form a hybridization product

20 that defines a gap opposite the selected nucleotide;

- b) adding an identified test nucleotide, a polymerase and a ligase, under conditions for polymerization and ligation;
- c) isolating the first hybridization product by contacting the first hybridization product with a capture oligonucleotide that is covalently coupled to a mobile

solid support, wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated second hybridization product; and

- 5 d) detecting the presence or absence of the fluorescent label, after dissociation of the hybridized nucleic acids, in the second hybridization product, the presence of the label indicating the polymerization of the test nucleic acid to the target oligonucleotide and ligation of the labeled reporter oligonucleotide to the target oligonucleotide linked to the mobile solid support, and the identity of the test
- 10 nucleotide indicating the identity of the nucleotide complementary to the selected nucleotide, thus identifying the selected nucleotide.

72. A method of quantitating expression of a selected nucleic acid in a sample comprising

- 15 a) contacting (i) message nucleic acid isolated from a selected source with (ii) a target oligonucleotide, wherein the target oligonucleotide comprises a first complementarity region and a second complementarity region 5' of the first complementarity region, wherein the first complementarity region comprises a region complementary to a section of the selected nucleic acid;
- b) performing a selected identification reaction with the first hybridization
- 20 product to determine the identity of the selected nucleotide wherein a selectively labeled detection product comprising the second complementarity region of the target oligonucleotide can be formed;
- c) isolating the detection product by contacting the detection product with a capture oligonucleotide that is covalently coupled to a mobile solid support,

wherein the capture oligonucleotide comprises a nucleic acid sequence complementary to the second complementarity region of the target oligonucleotide, under hybridization conditions to form an isolated hybridization product; and

- 5      d) quantitating the fluorescence in the isolated hybridization product, the quantity of fluorescence indicating the quantity of the selected nucleic acid in the sample.

73. The method of claim 72, wherein the message nucleic acid is mRNA, cRNA or cDNA.

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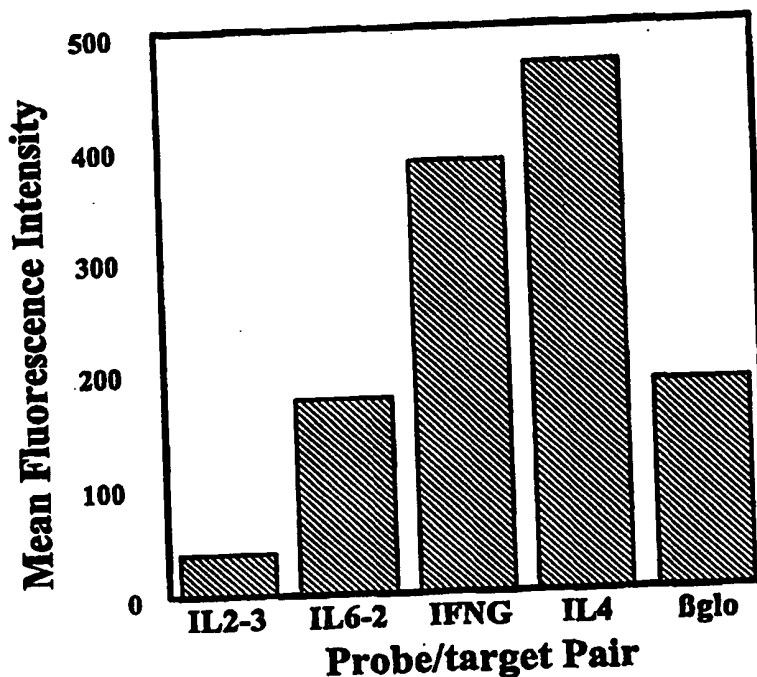
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>G01N 21/77, 21/64, C12Q 1/68</b>		A3	(11) International Publication Number: <b>WO 98/50782</b>
			(43) International Publication Date: 12 November 1998 (12.11.98)
(21) International Application Number: PCT/US98/09163		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 5 May 1998 (05.05.98)		Published With international search report.	
(30) Priority Data: 08/851,203 5 May 1997 (05.05.97) US		(88) Date of publication of the international search report: 4 February 1999 (04.02.99)	
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(54) Title: FIBER OPTIC BIOSENSOR FOR SELECTIVELY DETECTING OLIGONUCLEOTIDE SPECIES IN A MIXED FLUID SAMPLE

(57) Abstract

The present invention provides biosensors, apparatus and methods for selectively detecting at least one complementary oligonucleotide target specie in a fluid sample containing a mixture of different oligonucleotide fragments. One preferred embodiment of the biosensor is as a unitary fiber optic array having an in-situ hybridization zone comprising not less than one specie of single stranded oligonucleotide disposed as individual deposits in aligned organization upon multiple strand end faces at differing spatial positions on the distal array end surface. In this manner, a collective of deployed, single specie, multiple fixed probes are presented for selective in-situ hybridization on-demand with at least one mobile complementary target specie ultimately bearing a joined identifying label. The biosensor provides for optical detection of in-situ hybridization on the distal end surface via the presence of the concomitantly disposed joined identifying label at the differing spatial positions.



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# INTERNATIONAL SEARCH REPORT

Int. .lonal Application No

PCT/US 98/09163

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 G01N21/77 G01N21/64 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	see figure 23 ---	2,3,5-8
Y	US 5 244 636 A (WALT) 14 September 1993 cited in the application see abstract see column 4, line 53 - column 5, line 9 see column 5, line 58 - column 7, line 9 see column 8, line 40 - line 43 see column 24, line 34 - line 56 see column 26, line 3 - line 35 see figures 17,18 --- -/--	2,3,5-8

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Date of the actual completion of the international search

28 October 1998

Date of mailing of the international search report

05/11/1998

Name and mailing address of the ISA

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : <b>G01N 21/77, 21/64</b>	<b>A2</b>	(11) International Publication Number: <b>WO 98/50782</b> (43) International Publication Date: 12 November 1998 (12.11.98)												
(21) International Application Number: <b>PCT/US98/09163</b> (22) International Filing Date: <b>5 May 1998 (05.05.98)</b> (30) Priority Data: <b>08/851,203</b> <b>5 May 1997 (05.05.97)</b> <b>US</b> (71) Applicant (for all designated States except US): <b>TRUSTEES OF TUFTS COLLEGE [US/US]; Tufts University, Ballou Hall, Medford, MA 02155 (US).</b> (72) Inventors; and (75) Inventors/Applicants (for US only): <b>WALT, David, R. [US/US]; 4 Candlewick Close, Lexington, MA 02178 (US). HEALEY, Brian, G. [US/US]; 577 Nortontown Road, Guilford, CT 06437 (US). FERGUSON, Jane, F. [US/US]; Apartment 3, 111 Woodstock Street, Somerville, MA 02144 (US).</b> (74) Agent: <b>CREEHAN, R., Dennis; P.O. Box 750070, Arlington Heights, MA 02175-0070 (US).</b>		(81) Designated States: <b>AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</b>  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>												
(54) Title: <b>FIBER OPTIC BIOSENSOR FOR SELECTIVELY DETECTING OLIGONUCLEOTIDE SPECIES IN A MIXED FLUID SAMPLE</b>														
(57) Abstract <p>The present invention provides biosensors, apparatus and methods for selectively detecting at least one complementary oligonucleotide target specie in a fluid sample containing a mixture of different oligonucleotide fragments. One preferred embodiment of the biosensor is as a unitary fiber optic array having an in-situ hybridization zone comprising not less than one specie of single stranded oligonucleotide disposed as individual deposits in aligned organization upon multiple strand end faces at differing spatial positions on the distal array end surface. In this manner, a collective of deployed, single specie, multiple fixed probes are presented for selective in-situ hybridization on-demand with at least one mobile complementary target specie ultimately bearing a joined identifying label. The biosensor provides for optical detection of in-situ hybridization on the distal end surface via the presence of the concomitantly disposed joined identifying label at the differing spatial positions.</p> <div data-bbox="584 1155 1347 1827"><table border="1"><thead><tr><th>Probe/target Pair</th><th>Mean Fluorescence Intensity</th></tr></thead><tbody><tr><td>IL2-3</td><td>~50</td></tr><tr><td>IL6-2</td><td>~180</td></tr><tr><td>IFNG</td><td>~400</td></tr><tr><td>IL4</td><td>~480</td></tr><tr><td>Bglo</td><td>~210</td></tr></tbody></table></div>			Probe/target Pair	Mean Fluorescence Intensity	IL2-3	~50	IL6-2	~180	IFNG	~400	IL4	~480	Bglo	~210
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FIBER OPTIC BIOSENSOR FOR SELECTIVELY DETECTING OLIGONUCLEOTIDE  
SPECIES IN A MIXED FLUID SAMPLE

RESEARCH SUPPORT

5 The research for the present invention was supported by a grant from the National  
Institutes Of Health under contract grant GM 48142.

FIELD OF THE INVENTION

10 The present application is concerned generally with apparatus and methods for the  
analysis of genes and gene expression; and is particularly directed to the construction and  
use of a fiber optic biosensor able to detect selectively one or multiple DNA, RNA, or  
PNA oligonucleotide fragments concurrently.

BACKGROUND OF THE INVENTION

15 In less than twenty years, the field of molecular genetics, including the specialty of genetic  
engineering, has revolutionized the science of biology as a whole and is in the process of  
restructuring medicine in both diagnostic and therapeutic applications. Not only are  
individual genes now being isolated and characterized, but also extensive research studies  
as to how genes function and are regulated in-vivo are being actively pursued. Moreover,  
20 many techniques for manipulating and modifying genes have been reported and are today  
becoming widespread in use and diverse in application. Merely exemplifying the many  
authoritative texts and published articles presently available in the literature regarding  
genes, gene manipulation and genetic analysis are the following: Gene Probes for Bacteria  
(Macario and De Macario, editors) Academic Press Inc. 1990; Genetic Analysis. Principles  
25 Scope and Objectives by John R.S.Fincham, Blackwell Science Ltd., 1994; Recombinant  
DNA Methodology II (Ray Wu, editor), Academic Press, 1995; Molecular Cloning, A  
Laboratory Manual (Maniatis, Fritsch, and Sambrook, editors), Cold Spring Harbor  
Laboratory, 1982; PCR (Polymerase Chain Reaction), (Newton and Graham, editors), Bios  
Scientific Publishers, 1994; and the many references individually cited within each of these  
30 publications.

Among the many innovative ideas and novel techniques generated by molecular  
genetic research studies has been the generation of nucleic acid probes for identifying the  
existence of specific genes, the products of gene expression, and the presence of mutations

in one or more genes. By definition a nucleic acid probe is a DNA or RNA oligonucleotide fragment or peptide nucleic acid (PNA) of known base sequence. Existing as a single-stranded segment of base codons, a nucleic acid probe which will bind to a complementary base sequence of nucleic acids which is the analyte of interest for any purpose. Thus, the oligonucleotide probe, via its selective binding capability, is employed to detect and identify individual gene fragments or nucleic acid sequences present in viruses, bacteria, and other cells serving as samples for scientific, research or medical interest.

In general, any DNA, RNA, or PNA sequential fragment (obtained from any source and regardless of whether the sequence is naturally occurring or synthetically prepared) must meet two essential criteria in order to be truly useful as an oligonucleotide probe. First, the oligonucleotide probe sequence must be as specific as possible for the intended complementary target sequence; and, preferably, bind exclusively with only the complementary target sequence with little or no cross-reaction. Secondly, the oligonucleotide probe must be able to distinguish among closely related nucleic acid base sequences having a substantial degree of homology as well as be able to bind selectively with varying types and sources of nucleic acid fragments having the complementary target sequence as part of its composition. Thus, the size or length of the oligonucleotide probe and the repetitive nature of or copy number for the complementary target sequence will meaningfully affect not only the specificity, but the sensitivity of the probe for detection purposes.

The technique employing an oligonucleotide probe for selective binding to a complementary target sequence is generally termed "hybridization". However, the development of hybridization based assays for the identification of specific genes and gene expression products has been severely limited to date because of major difficulties in: (a) isolating highly specific nucleic acid sequences for use as oligonucleotide probes; (b) developing assay formats that are sufficiently rapid and simple in order to identify even one complementary target sequence in a fluid mixture containing many varieties of different single-stranded oligonucleotides in admixture; and (c) devising non-radioactive detection systems that provide a desired level of sensitivity. Thus, several types of DNA (or RNA, or PNA) hybridization assay formats have come into prevalent use.

Four hybridization assay formats are commonly employed today. Each of these hybridization detection formats suffers from relatively poor sensitivity, although various target sequence amplification techniques (such as PCR) have also been developed to

reduce the severity of this problem. The four most commonly used types of hybridization assay formats are: the Southern blot technique; the dot or spot blot technique; in-situ hybridization; and sandwich hybridization assays. As with the selection of an appropriate oligonucleotide probe, the choice of a hybridization assay format often rests upon the degree of specificity and sensitivity that is required for the particular analysis; and upon the factors of speed, reliability, and ease of performance and interpretation of the assay result - which varies markedly among the different assay formats.

In Southern blot assays, specimen DNA is isolated and purified prior to restriction endonuclease digestion; followed by separation of the digestion products by electrophoresis on an agarose gel, denaturation of the DNA in the gel, and transfer of the denatured DNA fragments to a solid matrix such as a nitrocellulose membrane. The DNA bound to the solid matrix is then hybridized in the presence of radioactively labeled DNA targets to establish homology between the probe and target DNA. Hybridization of the targets to the probes is detected by autoradiography and often requires several days or weeks of exposure. This format is thus often too lengthy and cumbersome for routine or large-scale analyses of many specimens.

The dot-blot procedure also requires that specimen DNA be isolated and purified before being denatured and applied to a suitable solid matrix (such as nitrocellulose). Hybridization to the matrix-bound DNA is then performed using probe-specific targets. The hybridization of target DNA to the probe DNA is detected either by auto-radiography or by visual inspection using non-radioactive detection procedures. The spot-blot assay format is similar except that specimens or specimen lysates are directly applied to the solid matrix without prior extraction of their DNA. Although this assay format allows many different samples to be processed at one time, these assays are often limited to high background noise that complicates the interpretation of results and is also subject to lengthy time of processing for each sample to be evaluated.

The in-situ hybridization technique intends that the DNA or RNA in the cells of a fixed tissue section or fixed culture cell be hybridized to DNA probes directly on a microscope slide. The results are determined by microscopy if non-radioactive detection systems are used and by autoradiography if radioisotopes are employed for the targets. This assay format can detect the presence of only a few copies of the target DNA sequence to be hybridized. The conventional in-situ hybridization assay is not suitable for screening large numbers of specimens due to the need to separate and remove extraneous cellular

materials from the sample prior to addition of the labeled target.

Lastly, the sandwich hybridization assay requires that at least two different specific probes hybridize to the target DNA of interest, rather than just one probe alone. In this format, the first probe (the capture sequence) is bound to a solid support and is allowed to  
5 bind (capture) the specimen DNA. A second probe (the signaling probe) with a sequence that is adjacent or close to the capture sequence on the target DNA molecule is then allowed to hybridize to the support-bound target DNA. This signaling probe can be labeled with either radioactive or non-radioactive labels; and the removal of non-specific cellular material in the first step of the procedure enhances the specificity of the hybridization  
10 assay by reducing the effects of contaminating tissue or debris.

More recently however, the value of using immobilized, spatially distinguishable, hybridization probes for concurrent analyses of multiple gene sequences has been recognized and resulted in the development of miniaturized hybridization assays using solid matrix assays [Southern, E.M., Trends in Genetics 12: 110-115 (1996)]. Thus,  
15 hybridization using said matrix arrays have been performed on glass surfaces [Maskos, U. and E.M. Southern, Nuc. Acids. Res. 20: 1679-1684 (1992); Guo et al., Nuc. Acid. Res. 22: 5456-5465 (1994)]; on microtiter plates [Kalakowski et al., Anal. Chem. 68: 1197-1200 (1996); Nikiforov et al., Nuc. Acids Res. 22: 4167-41 75 (1994); Rasumussen et al., Anal. Biochem. 198:138-142 (1991)]; on plastic sheets [Matson et al., Anal. Biochem.  
20 224:110-116 (1995)]; on thin polymer gels [Khrapko et al., J. DNA Seq. Map. 1: 375-388 (1991)]; and using semiconductor devices [Eggers et al., Bio Techniques 17: 51 6-524 (1994); Kreiner, T, Am. Lab.: 39-43 (1996)]. In addition, the desire for using non-radioactive means for detection have caused a surge of interest in means for detection of hybridization on solid matrix supports which employ fluorescence [Kumke et al., Anal. Chem. 67: 3945-3951 (1995); Piunno et al., Anal. Chim. Acta. 288: 205-214 (1994)];  
25 chemiluminescence [Ito et al., J. Neurosci. Methods 59: 265-271 (1995); Nguyen et al., Biosen. Bioelectron. 7: 487-493 (1995)]; evanescent wave technology [Graham et al., Biosen. Bioelectron. 7: 487-493 (1992); Strachan et al., Lett. App. Microbiol. 21: 5-9 (1995); Watts et al., Anal. Chem. 67: 4283-4289 (1995)]; confocal microscopy [Fodor et al., Nature (London) 364: 555-556 (1993)]; light scattering [Stimpson et al., Proc. Natl. Acad. Sci. USA 92: 6379-6383 (1995)]; electrochemistry [Milland et al., Anal. Chem. 66: 2943-2948 (1994); Pandey et al Anal. Chem. 66:1236-1241(1994) Hashimoto et al., Anal. Chim. Acta. 286: 219-224 (1994)]; and surface resonance phenomena [Yamaguchi et al.,

Anal. Chem. 65:1925-1927 (1993)].

Despite these recent innovations using probes immobilized on solid matrix arrays the major obstacles and limitations of hybridization methods generally continue to restrict and contain the currently available techniques and formats. These demands and limitations include a requirement for a large sample volume; an inability to perform multiple analyses concurrently in real time; a requirement for a relatively high concentration of target DNA (the complementary target sequence) in the fluid sample; an inability to detect multiple species concurrently; relatively slow kinetics for hybridization to occur between the target sequences and the immobilized probes within the assay format; and a dependence upon lengthy assays. Moreover, despite the use of new in-vitro amplification techniques such as the polymerase chain reaction procedure, the problems of assay sensitivity, lengthy times for analysis, the quantum of background signal noise, and the inability to detect more than one target nucleic acid sequence at a time remain as recurring handicaps and continuing obstacles for each of these techniques. It will be recognized and appreciated by persons working in this field today, therefore, that the development of a unique biosensor which overcomes and eliminates most, if not all, of these major limitations and procedural hindrances would be seen as a major advance and unforeseen improvement in this art.

#### SUMMARY OF THE INVENTION

The present invention has multiple aspects and formats. A first aspect provides an optical biosensor for selectively detecting an oligonucleotide specie in a fluid sample, said biosensor comprising:

a clad optical fiber strand of determinable configuration and dimensions which presents two strand end faces as discrete optic surfaces for introduction and conveyance of light energy;

an oligonucleotide in-situ hybridization zone comprising one specie of single-stranded oligonucleotide disposed as a deposit upon one of said strand end faces of said optical fiber strand, said deposit of single stranded oligonucleotide within said hybridization zone serving as a deployed, single specie, fixed probe suitable for selective in-situ hybridization on-demand with a mobile complementary oligonucleotide target specie,

(a) wherein such complementary oligonucleotide target specie as is hybridized in-situ ultimately bears a joined identifying label comprising at least one light

energy absorbing dye of known spectral characteristic, and

- (b) wherein the resulting, specie specific, in-situ hybridized oligonucleotide reaction product formed by a complementary oligonucleotide target specie with said fixed probe at said hybridization zone is optically detectable via the spectral characteristics of said joined identifying label concomitantly disposed at said strand end face surface.

A second aspect defines an optical biosensor for selectively detecting at least one oligonucleotide specie in a fluid sample, said biosensor comprising:

- a bundled array comprising a plurality of individually clad, fiber optic strands disposed co-axially along their lengths and having two discrete array ends each of which is formed of multiple strand end faces, said bundled array being of determinable configuration and dimensions and said two discrete array ends presenting two optic array surfaces for introduction and conveyance of light energy; and

- at least one oligonucleotide in-situ hybridization zone comprising a plurality of single stranded oligonucleotide species disposed as individual specie deposits in aligned organization at differing spatial positions on one zone of said optic array end surface of said bundled array, the differing spatial positioning for each deposit of single stranded oligonucleotide specie in aligned organization within said hybridization zone serving as deployed, single specie, multiple fixed probes suitable for selective in-situ hybridization on-demand with its mobile complementary oligonucleotide target specie,

(a) wherein such complementary oligonucleotide target specie as is hybridized in-situ ultimately bears a joined identifying label comprising at least one light energy absorbing dye of known spectral characteristics, and

- (b) wherein the resulting, specie specific, in-situ hybridized oligonucleotide reaction products formed by a complementary oligonucleotide target specie with said multiple fixed probes at said differing spatial positions within said hybridization zone are optically detectable via the spectral characteristics of said identifying label concomitantly disposed at said differing spatial positions.

- A third aspect of the present invention provides an optical biosensor for selectively detecting a plurality of individual oligonucleotide species concurrently in a fluid sample containing a mixture of different oligonucleotide species, said biosensor comprising:

a preformed, unitary fiber optic array comprising a plurality of individually clad, fiber optic strands disposed co-axially along their lengths and having two discrete optic



array ends each of which is formed of multiple strand end faces, said preformed unitary fiber optic array being of determinable configuration and dimensions and said two discrete optic array ends presenting two discrete optic array surfaces for introduction and conveyance of light energy; and

- 5 at least one oligonucleotide in-situ hybridization zone comprising a plurality of single-stranded oligonucleotide species disposed as individual specie deposits in aligned organization upon multiple strand end faces at differing spatial positions on one of said discrete optic fiber array surfaces of said unitary fiber optic array, the differing spatial positioning for each deposit of single-stranded oligonucleotide specie in aligned
- 10 organization within said in-situ hybridization zone serving as a collective of deployed, specie specific, fixed probes suitable for selective in-situ hybridization on-demand with its mobile complementary oligonucleotide target specie in a fluid mixture containing alternative mobile complementary target species,

- (a) wherein such hybridized complementary oligonucleotide target species as are
- 15 hybridized in-situ each ultimately bears a joined identifying label comprising at least one light energy absorbing dye of known spectral characteristics, and

- (b) wherein the resulting, species specific, in-situ hybridized oligonucleotide reaction product formed by a complementary oligonucleotide target specie with its collective of multiple fixed probes at said differing spatial positions within said
- 20 hybridization zone is optically detectable via the spectral characteristics of said joined identifying label concomitantly disposed at said differing spatial positions, each alternative in-situ hybridized oligonucleotide reaction product formed being concurrently optically detectable via the spectral characteristics of each joined identifying label at differing spatial positions within said hybridization zone.

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#### BRIEF DESCRIPTION OF THE FIGURES

The present invention may be more easily understood and better appreciated when taken in conjunction with the accompanying drawing, in which:

Fig. 1 is an overhead view of an individually clad, optical fiber strand;

- 30 Figs. 2A and 2B are views of the proximal and distal surfaces of the fiber optical strand of Fig. 1;

Figs 3A and 3B are alternative constructions of the optical end surface for the optical fiber strand of Fig. 1;

Fig. 4 is an overhead view of a bundled array of single core, optical fiber strands;

Figs. 5A and 5B are views of the distal and proximal end surfaces for the bundle of fibers of Fig 4;

Fig. 6 is an overhead view of a preformed, unitary fiber optic array using the optical fiber strand of Fig. 1;

Fig. 7 is a view of the intended distal array end surface of the unitary fiber optic array of Fig. 6;

Fig. 8 is a view of the intended proximal array end surface of the unitary fiber optic array of Fig. 6;

Fig. 9 is a frontal view of an illumination source able to provide light energy at precise spatial positions concurrently;

Figs. 10-15 illustrate the manipulative steps performed during the disposition of oligonucleotide probes at precise spatial positions on the distal array end surface of Fig. 8.

Fig. 16 is a schematic diagram of the apparatus comprising the biosensor

Fig. 17 is a schematic diagram of a fiber optic biosensor detection apparatus and system;

Figs. 18A and 18B are white light and background fluorescent images viewed through the proximal end of the biosensor apparatus;

Fig. 19 is a graph showing the background-subtracted mean fluorescence intensities obtained with fixed IL4 probes on the distal end of the biosensor;

Fig. 20 is a graph showing the plot of background-subtracted mean fluorescence as a function of time using a sensor having a  $\beta$ -glo probe and a 1.0  $\mu$ M f3-glo target solution;

Fig. 21 is a graph showing the plot of background-subtracted mean fluorescence as a function of time using a sensor having a  $\beta$ -glo probe and an 0.1  $\mu$ M f3-glo target solution;

Figs. 22A - 22F are fluorescent images from a biosensor apparatus after immersion in a IL2 target, a IL4 target, an IL6 target, a  $\beta$ -glo target, an IFNG target, a IL4 and IFNG and  $\beta$ -glo targets;

Fig. 23 is a graph illustrating the background-subtracted mean fluorescence signals as a function of the probe/target pair of Fig. 22;

Fig. 24 is a graph showing the plot of hybridization competition between labeled and unlabeled formats of the same target nucleic acid sequence;

Fig. 25 is a graph showing the fluorescence intensity of the poly(dA) matrix upon

repeated hybridization to and dehybridization from a poly(dT)-FITC target;

Fig. 26 is a graph showing the fluorescence intensity of a poly(dA)/acrylamide biosensor to varying concentrations of poly(dT)-F ITC;

Fig. 27 is a graph showing the calibration curve of the data of Fig. 25;

5 Fig. 28 is a graph showing the calibration curve of a poly(dA) matrix biosensor after in-situ hybridization with a poly(dT)-biotin target;

Fig. 29 is a graph showing the melting curves of a DNA biosensor after hybridization to a  $\Delta$  target at 28°C

10 Figs. 30A and 30B are fluorescent images from the DNA biosensor apparatus after in-situ hybridization with a  $\Delta$ -FITC target at 28°C and 50°C respectively;

Fig. 31 is a graph showing the fluorescence intensity over time for in-situ hybridization of a 196 nM  $\Delta$  target to a H-ras wt./H-ras  $\Delta$  matrix biosensor;

Fig. 32 is a graph showing the calibration of a DNA biosensor to a  $\Delta$ -FITC target at 54°C and

15 Fig. 33 is a fluorescence image from a DNA biosensor apparatus after a 20 minute hybridization time to a biotinylated  $\Delta$  PCR amplicon at 54°C followed by a 5 minute labeling reaction with streptavidin-FITC.

#### DETAILED DESCRIPTION OF THE INVENTION

20 The present invention is a fiber optic biosensor comprising at least one fiber optic strand; can comprise a bundled array of single core fibers joined together; and preferably comprises a preformed, unitary array of fibers -- these biosensor types having at least one and desirably several, oligonucleotide probes immobilized at individual and differing spatial positions on the distal end surface. The immobilized oligonucleotide probe(s) do  
25 not bear any identifying label of any kind. Rather, when the distal strand end surface or, alternatively, the distal array end surface supporting the immobilized probes are placed into reactive contact with a fluid sample containing at least one complementary target oligonucleotide segment bearing a joined identifying label, an in-situ hybridization will occur; and the reaction product of the in-situ hybridization (formed by a complementary  
30 oligonucleotide target collectively with the immobilized probe at each of the differing spatial positions on the distal end surface) can be detected and monitored by observing the fluorescence or reflected color of the joined identifying label that accompanied or is the consequence of hybridization at that specific spatial position. Equally important, because

the hybridization is specific between the oligonucleotide base sequence of the spatially immobilized probe with a complementary target base sequence, the detection of fluorescent or reflected light energy from the joined label at a predetermined spatial position on the distal end surface will demonstrate and evidence the occurrence of a specific and selective in-situ hybridization.

A number of different major advantages and unique capabilities are thus provided by the biosensor comprising the present invention. Among these advantages and capabilities are the following.

1. The present invention provides a biosensor which offers a choice of formats using a single optical fiber strand; or a bundled array of single core fibers joined together; or, alternatively, a plurality of optical fiber strands disposed co-axially along their lengths as a preformed unitary fiber optic array. The single optical strand format will provide one oligonucleotide hybridization zone and a single specie fixed probe suitable for selective in-situ hybridization with one mobile complementary oligonucleotide target specie. The bundled array of fibers format provides multiple hybridization zones and multiple single specie fixed probes for selective in-situ hybridization with one or a plurality of different mobile complementary oligonucleotide species. The alternative unitary fiber optic array format provides collectives of multiple and different fixed sets of specie-specific probes, each set of fixed probes being collectively suitable for selective in-situ hybridization with a different mobile complementary oligonucleotide target specie. The unitary fiber optic array format and the bundled array of fibers format are thus able to detect multiple and diverse types of mobile oligonucleotide target species simultaneously or concurrently in one sample and a single test; in comparison, single optical fiber strand format is limited to the detection of a single oligonucleotide target specie per sample and test.
2. The present invention, in all formats, is able to detect one specific target nucleic acid segment in a fluid mixture of different oligonucleotide fragments; and, in two formats, is able to detect a plurality of different complementary target oligonucleotide species in a single fluid sample containing a mixture of many different nucleic acid sequence fragments. The specificity of the in-situ hybridization reaction depends primarily upon the choice of oligonucleotide probe employed. Thus, when probes are chosen which have a known base sequence and having a recognized or specified binding capability, the hybridization is highly selective and specific for a single complementary nucleic acid target sequence; and only selective binding to that one complementary species will occur

despite the presence of other oligonucleotides in the fluid sample. Similarly, multiple and different in-situ hybridization reactions can concurrently occur at different spatial positions on the distal array end surface using a variety of alternative fixed probes. Each fixed probe is species specific; is fixed at a predetermined spatial position on the distal end surface; and will hybridize in-situ on-demand with its intended complementary target specie.

3. The present invention provides a biosensor which is extraordinarily rapid in providing evaluations and results based on in-situ hybridization reactions. Typically, the biosensor will provide optical detection of a labeled complementary target specie in a fluid sample in less than ten (10) minutes time and depending on the chosen components of the detection system, may provide optical detection of multiple target species concurrently or simultaneously within the same time duration, approximately ten minutes or less. The present invention thus provides a speed of detection which is unmatched by any previously known format or conventional technique.

4. The present invention provides an extremely high level of sensitivity for in-situ hybridization reaction. As the experimental data provided hereinafter reveals, even a simple embodiment of the biosensor and its supporting apparatus can optically detect 10 nM of a target oligonucleotide segment via the spectral characteristics of a joined identifying label. The apparatus and method of optical detection yields very little background signal as noise; and it is expected that as little as 0.01-0.1 nM will be detectable under optimum operating conditions. Accordingly, the traditional requirement for high concentrations of DNA (or RNA, or PNA) test sample in order that an accurate and reliable measurement be made is no longer necessary or required. Also, the absolute amounts of DNA (or RNA, or PNA) needed for detection are small due to the extremely small size of the sensor.

5. The present invention will provide high specificity of in-situ hybridization reactions in a fluid sample when used at ambient room temperatures generally, or at elevated temperatures. No special or unique environmental considerations or demands are necessary in order to use the present invention; to the contrary, the sole requirement is that the distal end surface bearing the specie-specific fixed probes be placed in reactive contact with a fluid sample suspected of carrying the complementary target base sequence.

6. The present invention requires only a very small fluid sample volume in order for optical determinations to be made and accurate results to be obtained. If necessary, a few nanoliters of fluid may be employed for detection purposes. It is generally desirable,

however, that a 1-2 microliter volume or greater be employed as the fluid sample. This low volume feature for the fluid sample may be maintained so long as there is sufficient liquid volume to cover the distal end surface effectively such that the fixed probe(s) immobilized on the distal surface may come into reactive contact with the contents of the fluid sample  
5 itself. If this minimal requirement is met and satisfied by the user, the true volume of the fluid sample is irrelevant and inconsequential.

7. The present invention also allows the user the ability to monitor the hybridization process over time in-situ without physical separation of the apparatus from the fluid sample undergoing evaluation. Thus, assuming the user employs a very rapid reaction time  
10 for analysis (less than 10 minutes), continuous observation and optical detection of the ongoing hybridization process over the 10 minutes can be made for one or for all of the differing spatial positions for the fixed probes immobilized on the distal end surface. Thus as hybridization occurs at each fixed probe location on the end surface, the presence of a bound label joined directly or indirectly to the complementary target specie will be  
15 detected via the spectral characteristics of the light energy absorbing dye employed as an identifying label. The user may thus monitor the ongoing reaction in real time as it actually proceeds in-situ; and may determine even more quickly whether or not the complementary target specie does in fact exist within that fluid sample.

8. The biosensor of the present invention allows the user not only to detect rapidly the  
20 presence of the complementary target base segment in a fluid sample, but also permits the user to quantify in proportional degree the concentration of the complementary target specie actually present in the fluid sample volume. A semi-quantitative estimate is based on a calibrated detection of the signal intensity emanating from the fixed probes undergoing in-situ hybridization with the fluid sample. In addition, a competitive assay in  
25 which a fixed amount of added labeled target specie is displaced proportionately by an unknown quantity of unlabeled target in a test sample will also provide quantitative results. A quantitative estimate which is both reliable and reproducible is rarely otherwise available without much more rigorous experiments and analytical test conditions.

For easier comprehension and a better appreciation of the features and attributes of  
30 the present invention, the detailed disclosure herein will be presented in separate sections seriatim. The order of presentation will be: a detailed description of the unitary fiber optic array comprising the biosensor; the DNA (or RNA, or PNA) oligonucleotide(s) which may be employed as probes; a preferred means for immobilizing the oligonucleotide probes at

differing spatial positions on the distal array end surface; the supporting apparatus desirably employed with the biosensor for detecting in-situ hybridization reactions on-demand; a description of complementary nucleic acid segments as target species; the manner of identifying the resulting in-situ hybridized reaction product; and a series of experiments and empirical results demonstrating some of the major advantages and unusual benefits provided by the present invention. Each of these will be described in detail below.

Also, since the present invention is definable in multiple formats and may be employed in different modes for a variety of divergent purposes and applications, the subject matter as a whole which is the present invention will be presented and described individually as component parts and then collectively as assemblies in order that the prospective user may more quickly recognize and appreciate their major differences and distinctions in comparison to the conventionally known systems.

#### I. The Organization And Construction Of The Biosensor

The unique biosensor may be prepared in three different formats. The simplest format employs a single optical fiber strand; and presents a single oligonucleotide in-situ hybridization zone disposed on the distal end surface comprising a single species of single-stranded DNA (or RNA, or PNA) disposed as a fixed probe for selective hybridization with one mobile complementary target species. A more elaborate format is the bundled array of single core fibers joined together. This format type presents an in-situ hybridization zone; and allows the user a choice of employing either a single species or multiple species of DNA (or RNA or PNA) as fixed probes for selective in-situ hybridization. The alternative and most sophisticated format employs: a preformed, unitary fiber optic array comprised of a plurality of individually clad fiber optical strands disposed co-axially along their lengths; and at least one oligonucleotide in-situ hybridization zone comprising not less than one species of single-stranded DNA (or RNA, or PNA) disposed as a plurality of individual deposits in aligned organization upon multiple fiber optical strand end faces at differing spatial positions and which serve as a collective of multiple fixed probes for species selective in-situ hybridization on-demand. Each format will be described in detail.

## A. The Format Choices

The individually clad, optical fiber strand

A typical optical fiber strand is illustrated by Figs. 1 and 2A and 2B. As seen therein, an individual optical fiber strand 10 is comprised of a single optical fiber 12 having a rod-like shaft 14 and two fiber ends 16, 18, each of which provides a substantially planar end surface. The intended distal surface 20 at the fiber end 16 is illustrated by Fig. 2A, while the intended proximal surface 22 at the fiber end 18 is illustrated within Fig. 2B. It will be recognized and appreciated that the terms "proximal" and "distal" are relative and interchangeable until the strand is ultimately positioned in an apparatus. The optical fiber 12 is composed typically of glass or plastic; and is a flexible rod able to convey light energy introduced at either of its ends 16 and 18. Such optical fibers 12 are conventionally known and commercially available. Alternatively, the user may himself prepare individual optical fibers in accordance with the practices and techniques reported in the scientific and industrial literature. Accordingly, the optical fiber 12 is deemed to be conventionally known and available as such.

It will be appreciated that Figs. 1-2 are illustrations in which the features have been purposely magnified and exaggerated beyond their normal scale in order to provide both clarity and extreme detail. Typically, the conventional optical fiber has a cross section diameter of 5-500 micrometers; and is routinely employed in lengths ranging between meters (in the laboratory) to kilometers (in field telecommunications). Moreover, although the optical fiber 12 is illustrated via Figs. 1-2 as a cylindrical extended rod having substantially circular proximal and distal end surfaces, there is no requirement or demand that this specific configuration be maintained. To the contrary, the optical fiber may be polygonal or asymmetrically shaped along its length; provided with special patterns and shapes at the proximal and/or distal faces; and need not present an end surface which is substantially planar. Nevertheless, for best efforts, it is presently believed that the substantially cylindrical rod-like optical fiber having planar end surfaces is most desirable.

Each optical fiber 12 is desirably, but not necessarily, individually clad axially along its length by cladding 26. This cladding 26 is composed of any material with a lower refractive index than the fiber core and prevents the transmission of light energy photons from the optical fiber 12 to the external environment. The cladding material 26 may thus be composed of a variety of radically different chemical formulations including various glasses, silicones, plastics, platings, and shielding matter of diverse chemical composition



and formulation. The manner in which the optical fiber 12 is clad is also inconsequential and of no importance to the present invention. Many methods of deposition, extrusion, painting and covering are scientifically known and industrially available; and any of these conventionally known processes may be chosen to meet the requirements and convenience of the user. Moreover, the quantity of cladding employed need only be that minimal amount which effectively prevents light energy conveyed by the optical fiber 12 from escaping into the ambient environment. It will be recognized and appreciated therefore, that the depth of cladding 26 as appears within Figs. 1 and 2 respectively is greatly exaggerated and purposely thickened in appearance in order to show the general relationship; and is without scale or precise ratios between the cladding 26 and the optical fiber 12.

It will also be recognized that the configuration of the cladding 26 as shown by Figs. 1 and 2 has been shaped as a circular coating to illustrate a preferred embodiment only. For reasons as will become clear subsequently, it is desirable that the cladding 26 take form in regular geometric form such as a round or circular shape. The illustrated configuration, however, is merely a preferred embodiment of the cladding 26 as it extends co-axially along the length of the optical fiber 12. For purposes of added clarity also, Fig. 1 reveals the individually clad, optical fiber strand 10 in partial cross-section to demonstrate the relationship between the optical fiber core 12 and the cladding 26 which is coextensive along its length.

The user also has a variety of choices at his discretion regarding the configuration of the "distal" end 16 of the optical fiber 12 as shown by Figs. 3A and 3B; however, both ends of the strand must be the same -- i.e., if the "distal" end is cylindrical then the "proximal" end must be also. As seen in Fig. 3A, the "distal" end 16 is substantially cylindrical in shape and desirably presents a surface 20 which is substantially planar and smooth. A possible, but less desirable, alternative is shown by Fig. 3B, in which the distal end 30, nevertheless provides a very different end surface for the optical fiber 12. The surface 32 includes a depression or well 34 which extends into the substance of the optical fiber 12 at a depth typically of several micrometers. Although the well 34 appears substantially circular within Fig. 3B, oval or other rotund configured depressions may also be employed as fits the needs or convenience of the use. Similarly, the void volume of the well 34 from its greatest depth to the proximal surface 32 may also be varied.

It will be recognized and appreciated as well that the range and variety of

dimensional and configurational divergence for the strand end is limited only by the user's ability to subsequently dispose and immobilize an oligonucleotide of known composition/formulation of controlled thickness on the intended distal surface of the optical fiber 12. In some embodiments, a greater depth of deposit on the surface of the distal end surface may be highly desirable; nevertheless, for most general assay purposes, both quantitative and qualitative, the intended distal surface illustrated within Fig. 3A as a substantially planar and smooth surface is deemed to be most suitable and desirable.

For general construction of the single strand sensor and for most purposes and applications of the improved optical detecting system and procedures described hereinafter, it is desirable to employ the individually clad, fiber optical strand illustrated by Figs. 1, 2A, 2B in preference to a bare, unsheathed strand. Clearly, the optical fiber strand is unable to transmit light energy photons to any other entity due to the cladding material 26 which forms a sheath. This sheath, having a refractory index less than the strand core, also prevents loss of light energy photons into the general environment. Accordingly, the potential for photon loss, distortion, or other optical error is minimized and reduced. For these reasons, the individually clad optical fiber mode of construction is preferable to the use of bare optical fiber strands in order to achieve greater precision and accuracy.

#### The bundled array of single core fibers

It will be noted that the bundling of individual single core fibers into an organized array can be made either before or after one oligonucleotide specie has been deposited and immobilized on the distal end surface of each optical fiber strand forming the bundled array of fibers. A detailed and empirically evaluated bundled array of fibers prepared in advance and used herein as a biosensor is presented by the data of Experimental Series A comprising Experiments 1-5 respectively.

For illustrative purposes and ease of understanding, however, the detailed description in this section will limit itself to a bundled array of fibers which do not yet have any oligonucleotide specie immobilized onto their individual strand end faces. In addition, the number of individual, single core, optical fibers employed in a bundled array format has been intentionally limited to only three strands. It will be recognized and appreciated, however, any small quantity of single core optical fiber strands ranging in number from not less than 2 to about 20 fibers can be combined together as a single discrete bundle, the exact number of single core strands being bundled into an array

depending greatly upon the size of the core strands.

The bundling of individual, single core, optical fiber strands into an organized array is a conventionally known technique and is well established in this field. A number of different bundling formats are well known which combine prepared individual single strand sensors as an integrated bundle for a specific purpose. Merely exemplifying the conventionally known range of articles using the bundled array of fibers format are European Patent Application 88105676.6; and U.S. Patent Nos. 5,047,627 and 4,999,306, as well as the references cited within each of these.

Accordingly, a typical bundled array of single core optical fibers is illustrated by Figs. 4 and 5 respectively. As seen therein, the bundled array 40 formed of single core fibers appears as a substantially triangular-shaped integrated article having a distal end face 42 and a proximal end face 44; and is comprised of three different and distinct, single core, optical fiber strands 50, 60, and 70 which are disposed co-axially along their lengths to form the integral bundle. Each of the individual strands 50, 60, and 70 is a single optical fiber strand having a distal strand end face 52, 62, 72 and a proximal strand end face 54, 64, 74. Also, each strand has a single optical core 56, 66, and 76 which is individually covered and surrounded by cladding material 58, 68, and 78.

By way of example, as shown in Figs. 4 and 5, three single core fibers 50, 60, 70 are shown joined together as a configured bundle using epoxy adhesives which are optically dense and using such additional cladding material as is necessary as a bulk filler to form an integral fiber bundle 40 configured in substantially triangular form. A sheath 41 may optionally be used to protect the unexposed length 43 of the bundle. Note that each optical fiber strand in the bundled array 40 has been spatially oriented and intentionally placed into a triangular configuration such that fiber strand 50 lies at the apex while fiber strands 60 and 70 lie at the base of the shaped bundle. Once placed and immobilized into this triangular organizational format, the spatial positioning for each of the three single core fibers 50, 60, and 70 within the bundled array 40 is always maintained.

Furthermore, Fig. 5A shows that the distal ends 52, 62, 72 of each respective single core strand 50, 60 and 70 each individually extends for a short distance (typically several centimeters) beyond the protective sheath 41. This is a desirable feature which facilitates the subsequent immobilization of one oligonucleotide specie to the distal strand end of each face 52, 62, and 72 respectively. In comparison, Fig. 5B shows that the proximal end face 44 of the bundled array 40 is coextensive with the proximal strand end faces 54, 64,

and 74 of the single core fibers 50, 60 and 70 respectively.

#### The unitary array

A typical preformed fiber optic array, its organization and construction, and its  
5 component parts are illustrated by Figs. 6-8 respectively. Each discrete unitary fiber optic  
array is a preformed composite comprised of a plurality of individually clad, fiber optical  
strands disposed coaxially along their lengths. The smallest common repeating unit within  
the preformed array is thus a single optical fiber strand. The manner in which these optical  
fiber strands are prepared and the manner in which these prepared optical strands are  
10 joined collectively into an organized optic array are conventionally known, but is  
fundamental to a proper understanding and use of the alternative format.

It is recognized also that the unitary array of optical fibers has been used previously  
as a major component in other inventions and is described in earlier issued patents. Such  
usage is exemplified by U.S. Patent Nos. 5,244,636; 5,244, 813; 5,250,264; and 5,298,741;  
15 the texts of which are individually expressly incorporated by reference herein.

The unitary fiber optic array 100 appears in exaggerated, highly simplified views  
without regard to scale within Fig. 6. The preformed array is composed of a plurality of  
individually clad, fiber optical strands which collectively lie coaxially along their  
respective lengths as the discrete, unitary optic array 104 of fixed and determinable  
20 configuration and dimensions. The optic array 104 has a unitary, rod-like collective body  
106 and intended distal and proximal collective ends 108,110 formed of multiple strand  
end faces. The intended distal collective end 108 provides a substantially planar and  
smooth optic array surface 114. The topographical surface 116 is the result of fusing the  
clad of each fiber optical strand 102 collectively with a fiber material 118 such that the  
25 fusion is drawn and appears as a discrete, unitary array. In this manner, the exterior surface  
116 of the collective array body 106 may be configured and dimensioned as an assembly in  
an acceptable manner and useful manner. It will be recognized and appreciated also that a  
substantially cylindrical configuration and topography is maintained and presented by the  
unitary imaging fiber optic array 100 merely as one preferred embodiment. Any other  
30 regular or irregular configuration and design may be achieved and employed to satisfy the  
individual user's needs or desires.

For purposes of clarity and ease of understanding Figs. 7 and 8 present a very  
limited and greatly reduced number of individually clad, fiber optical strands 102 present

within the preformed optical array 104. A total of only 120 individually clad, fiber optical strands are seen to comprise the optical array 104 in greatly magnified and scale-exaggerated views. Moreover, the relationship of the optical array surface 112 (the intended distal end) with respect to the other optical array surface 114 (the intended proximal end) becomes simplified and more-readily appreciated when using this limited number of 120 optical fiber strands. In practice and reality, however, it is estimated that typically there are 2000-3000 optical fiber strands in a conventional array of 200  $\mu$ m diameter. Thus the true total number of individually clad, fiber optic strands forming the unitary imaging fiber optic array will typically be in the thousands and vary substantially with the cross-sectional diameter of each optical fiber and the thickness of the cladding material employed when constructing the optical fiber strands themselves.

The construction, coherent organization, and positional alignment within a typical fiber optic unitary array is revealed by Figs. 6-8. For descriptive purposes only, each of the individually clad, optical fiber strands is presumed to be linearly straight in position and has been arbitrarily assigned an identifying number S1-S120 as shown via Figs. 7 and 8. The intended distal optical array end surface 112 of Fig. 7 shows that each of the individual optical fiber strands S1-S120 can be identified and distinguished from its adjacently disposed neighbor as well as from any other optical fiber strand within the preformed array 104 by a set of spatial positioning coordinate numbers for the strand end faces. The intended distal optical array surface 112 may be arbitrarily divided into two axial directions as is illustrated by Fig. 7. The exact location of the S1 strand is thus identifiable by the numerical coordinates "XII D" showing the strand end face. Similarly, the exact spatial positioning and strand end face of the S72 fiber is designated as "VIM." In this manner, the individual spatial position and strand end faces for each optical fiber strand S1-S120 is thus completely locatable and identifiable using the coordinate numeral labeling system.

The other optic array end surface 114 (the intended proximal end surface) allows for a similar mode of identification (presuming straight linear alignment of strands) by spatial positioning of each individual optical strand -- again as a result of using dual-axis numerical coordinates as seen in Fig. 8. Accordingly, fiber end strand end face S1 is located at numerical position "12d", and fiber S72 is identifiable, locatable, and distinguishable from all other fibers at the optic array surface by its individual numerical coordinates "6m". In this manner, the precise and exact position of each individually clad

optical fiber strand and strand end faces on each of the discrete optic array surfaces 112,114 can be located, identified, and specified via a series of two different numerical coordinates. The intended distal and proximal optic array surfaces are thus completely identifiable and distinguishable as per individual fiber optical strand 102 despite its  
5 presence in the preformed collective body 106 of the unitary fiber optical array 100.

It will be appreciated also that the overall organization of the individually clad, optical fiber strands 102 within the unitary array 100 of Figs. 6-8 is as aligned, parallel, strands which maintain their relative organizational positioning in a coherent, consistently aligned manner over the entire length of the collective body 106. This is deemed to be the  
10 most desirable and most easily constructable organization scheme for the preformed optical fiber array of the present invention.

Although this highly organized, coherent, and rigidly aligned collective construction is deemed to be most desirable, this high degree of organizational alignment is not an absolute requirement for each and every embodiment using an unitary optical array.  
15 Alternative manufacturing practices allow for a more random disposition of the individually clad, optical fiber strands disposed coaxially along their lengths. Although less desirable, a partially random disposition and a completely random alignment of the optical fiber will also result in a unitary collective body of optical fibers and in proximal and distal collective ends which provide two discrete optical array surfaces. It will be recognized  
20 therefore that while the individually clad, optical fiber strands may lie adjacent one another at one end, they may deviate and meander through the length of the array such that their position relative to one another may vary substantially in part or in whole -- thereby creating semi-coherent or incoherent positional alignments which vary in the randomness of their organizational construction. There is no requirement that the positioning of the  
25 intended proximal end of one strand be aligned and/or identical with the positioning of the intended distal end within the unitary optic array.

The entirety of the construction for the unitary optical fiber array (whether uniformly coherent, semi-random, or completely randomly organized) provides a means of introducing light energy photos of any determinable wavelength at one optic array surface  
30 with the knowledge that the light energy will exit at the other optic array surface. Therefore, by using the preferred completely coherent and rigidly maintained parallel alignment of strands illustrated by Figs. 7 and 8 (the intended distal and proximal optic array end surfaces respectively) of a unitary fiber optic array, the user may introduce light

energy to a portion or all of the optic array end surface 114 and have accurate knowledge and confidence that the light energy would be conveyed by the fiber strands and exit from the other optic array end surface 112. Conversely, were light energy introduced to the optic array end surface 112, the light energy would be conveyed by the optical fibers of the array and will exit from the other optic array end surface 114.

In addition, the topography of the unitary optic array end surfaces 112 and 114 will vary with the nature of the end faces for the individual optical fibers strands comprising the array. Thus, if the optical fiber strand end faces conform to that illustrated by Fig. 3A, then the array end surface will present a substantially planar and smooth topography.

Alternatively, however, if the optical fiber end faces forming the array are exemplified by Fig. 3B; then the unitary array end surface will appear as a collective of wells or depressions, each well extending into the collective substance of the array end surface at a set depth (typically of a few micrometers). The topography of the unitary array end would then present a pitted and crater-like surface for the immobilization of oligonucleotides as fixed probes.

It will also be recognized that the user may chose to introduce light energy to only a specific spatial location on the optic array end surface 114 -- for example, only to fibers S1, S7 and S8 -- and have accurate knowledge and confidence that the light energy would be conveyed only by those three optical fiber strands and exit from numerical positions "XIID", "XIC", and "XID" alone on the optic array end surface 112. No other light energy would appear from any other spatial position from the optic array surface 112. Similarly, were light energy of specific wavelength introduced at the optic array surface 112 via fibers S107, S108, and S115, respectively, the use can accurately predict and identify that the light energy will be conveyed by only these three optical fibers; and will exit only at the optic array surface 114 of numerical coordinate position numbers 2c, 2d, and 1d respectively and from no other spatial positions on this optic array surface. In this manner, not only does one have knowledge of the individual spatial positioning of each optical fiber strand in the preformed array but also one has the ability to identify and precisely locate light energy photons emerging from individual optical fiber strands within the whole of the optic array surface in a practical and reliable mode.

Accordingly, the critical and essential requirements of any optical fiber array construction allows and demands the capability for precise spatial positional introduction and conveyance of light energy via different fiber optical strands within the collective body

of the preformed, unitary fiber optical array. This capability to introduce light energy photons at precise spatial positions at one optic array of a unitary array; to convey the introduced light energy along the length of only a few fiber optical strands; and to control the exit of the conveyed light energy at a second, precisely known, spatial position on the other optic array surface of the unitary array is a hallmark of the singular fiber optic sensor presented herein.

B. Oligonucleotides Immobilized As One Or As Multiple Fixed Probes

The construction of the biosensor intends that at least one specie of single-stranded DNA, or RNA, or PNA, fragment be deployed and immobilized on the distal end surface of a single optical fiber strand; or on the distal end surface of at least one single core strand comprising an organized bundled array of optical fibers; or on the distal array end surface of a unitary array at differing spatial positions -- and thus serve as one or more deployed fixed probes suitable for selective in-situ hybridization on-demand. Each specie of DNA (or RNA, or PNA) fragments is disposed as an individual deposit on at least one strand end face or as multiple independent deposits in aligned organization on multiple strand end faces at one or at many different spatial positions on the distal optic end surface. Each oligonucleotide specie deposit on an optical fiber strand end face, therefore, serves as one fixed probe immobilized at a predetermined spatial position; and in each of the array formats, the multiple fixed probes serve collectively as many singular and different reaction zones acting repetitiously and in common for in-situ hybridization with a mobile complementary nucleic acid target (directly or indirectly bearing a joined identifying label).

25 The nature of the oligonucleotide fragment

The oligonucleotide sequence chosen for use as a probe may be obtained from any source; prepared or purified in any manner; vary in size or length without meaningful limit; and be used as a genetic tool for any research purpose, for a diagnostic goal or therapeutic application, or for any other result involving genetic manipulation. The chosen nucleic acid sequence may be DNA, or RNA, or a peptide nucleic acid (PNA) in composition; may be endogenously or exogenously derived; may be naturally occurring or synthetically prepared; may be composed of a minimum number of nucleic acid bases; or be composed of many thousands of nucleotides in sequence. For example, the user may thus employ



genomic or chromosomal DNA, plasmid DNA, and/or a cloned or replicated DNA which is representative of a marker or is unique for a virus, a bacteria, a particular cell type, or a cytoplasmic or nuclear zone within a cell. Similarly, the oligonucleotide or peptide nucleic acid fragment utilized may represent or comprise RNA in its many forms (such as messenger or transfer, or mitochondrial RNA) as it exists in nature or is synthetically produced. All of these diverse origins and sources of nucleic acid sequences are conventionally known; and many techniques for their individual preparation, isolation, and purification as single-stranded nucleic acid chains of known composition, sequence, and specific binding capabilities are available in the published literature today.

Regardless of the particulars regarding the nucleic acid sequence or fragment chosen for use as an oligonucleotide probe, two essential requirements must be met and satisfied in every instance. First, the nucleic acid segment should be of known composition and sequence order, and thus bind selectively and specifically with a particular complementary target sequence primarily, if not exclusively. The degree of selectivity and hybridization for the oligonucleotide probe will thus vary with the specificity of its nucleic acid sequence and the degree of homology permitted among different and competing complementary target species. Thus, the more controlled the nucleic acid sequence of the probe, the more selective and specific the binding with one complementary target specie. Second, the oligonucleotide probe must bind with its intended target at each and every instance and occasion where the complementary target species is presented for reactive contact. Thus, although the complementary target sequence may exist in single or low copy number and/or be encased in a larger-sized fragment containing non-complementary sequences, the oligonucleotide probe should nevertheless bind to the target portion of these larger mobile fragments when they come into reactive contact with the probe. Such binding capacity provides both specificity and sensitivity for the biosensor as a whole.

### C. Oligonucleotide Probe Immobilization

When depositing the individual DNA or RNA oligonucleotide specie to be used as a probe on the end surface of a single optical fiber strand or at precisely spatially positioned locations on one optical array end surface, it is necessary that the oligonucleotide(s) remain immobilized at the single or the different spatial positions assigned to each of them individually without migrating towards any other position. Multiple methods of oligonucleotide probe deposition and immobilization are

conventionally known and are suitable for use in making an embodiment of the present invention. Thus, one may prepare a specific formulation comprising one specie of DNA (or RNA, or PNA) bases in sequence and dispose the formulation at a specific spatial position and location on the optic end surface.

5        Among the conventional practices of deposition a variety of generally applicable polymerization processes are known, including thermal techniques, ionization methods, plasma methods, and electroinitiation procedures. These different methodologies are exemplified by the following publications, the text of each being expressly incorporated by reference herein. Thermal methods: Graham et al., J. Org. Chem. 44: 907 (1979); Stickler  
10 and Meyerhoff, Makromol. Chem. 179: 2729(1978); and Brand et al., Makromol. Chem. 181: 913(1980). Ionization methods: A. Chapiro, Radiation Chemistry of Polymer Systems, Chapter IV, Wiley-Intersciences, Inc., New York, 1962; JE. Wilson, Radiation Chemistry of Monomers, Polymers, and Plastics, chapters 1-5, Marcel Dekker New York, 1974. Plasma methods: Yasuda, W. and T.S. Hsu, J. Polym. Sci. Polym., Chem. Ed. 15:  
15 81(1977); Tibbett et al., Macromolecules 10: 674 (1977). Electroinitiation method: Pistoria, G. and O. Bagnarelli, J. Polym. Sci. Polym. Chem. Ed. 17: 1001 (1979); and Philips et al., J. Polym. Sci. Polym. Chem. Ed. 15: 1563 (1977).

One method of oligonucleotide probe disposition and immobilization preferred for use with unitary fiber optic arrays is the process known as photoactivation; and employs  
20 one or more photoactivated monomer preparations in admixture with one species of oligonucleotide as a photopolymerizable formulation [as described in Munkholm et al., Anal. Chem. 58: 1427 (1986) and Jordan et al., Anal. Chem. 59: 437 (1987)]. Such monomer preparations typically comprise solutions of several monomers in admixture and a concentration of the chosen DNA or RNA oligonucleotide specie. A representative  
25 listing of different monomer compositions suitable for preparing an admixture which subsequently can be photopolymerized are given by Table 1 below.

It will be appreciated that the listing of Table I are merely representative of the many different substances which can be usefully employed in admixture with a specie of oligonucleotide. In addition, the scientific and industrial literature provides many  
30 alternative monomer preparations and admixtures which are also suitable for use in making the present invention. Accordingly, all of these conventionally known monomer preparations are considered to be within the scope of the present invention.

Table I5 A. Monomers

acrylamide

N,N-methylene bis (acrylamide)

hydroxyethylmethacrylate

10 EGDMA

vinyl acetate

N-(3-aminopropyl) meth-acrylamide

hydrochloride [Kodak, Inc.]

N-acryloxy succinimide

## II. A Preferred Method Of Making A Biosensor

To demonstrate a most desirable method of making the biosensor comprising the present invention; and as a demonstration of the effectiveness for making optical determinations using the fully constructed fiber optic sensor, a detailed description of the manipulative steps for making a sensor able to hybridize in-situ with and consequently  
5 detect a mobile complementary oligonucleotide target specie bearing an identifying dye label is presented. It will be expressly understood, however, that the detailed description which follows hereinafter is merely illustrative and representative of the many different kinds of biosensors utilizing a unitary fiber optic array which can be made having one or  
10 more individual species of oligonucleotides deposited as multiple fixed probes at precise spatial positions on the optical array end surface, each disposed species-selective fixed probe being able to react with and individually hybridize with a labeled, mobile complementary target specie of oligonucleotide which is of interest in a fluid sample.

### 15 Surface silanization

Initially, fiber optic array similar to that illustrated by Figs. 6-8 respectively was obtained from commercial sources [Applied Fiber Optics, Inc., Southbridge, Mass.]. One optical array surface was submerged in a acetone 10% solution of 3-(trimethoxysilyl) propyl-methacrylate dispersed in dry acetone and allowed to soak for 2 hours duration.  
20 After silanization, this optical array surface was rinsed first with dry acetone and then with distilled water.

### Light Source

A fiber optic connector and ferrule [AMP, Inc., Harrisburg, Pa.] were modified to  
25 physically secure the fiber optic array to a fiber optic cable able to transport light energy of varying wavelengths to precise spatial positions on the distal array surface of the imaging fiber optic array. The exterior surface of one representative lighting cable is illustrated in an enlarged view by Fig. 9.

30 An inspection of the lighting cable of Fig. 9 reveals (in an exaggerated, highly oversimplified view for purposes of clarity) that the individual light sources via coordinated numerals correspond precisely to the spatial positions of Figs. 7 and 8; and are directly aligned with individual fiber optical strands S1-S120 (which also are precisely

positioned spatially and identifiable via linear coordinates). Thus, light originating from source L1 will be introduced only to fiber S1 spatially positioned at coordinate number "12a"; similarly, light energy emanating from source L85 will be introduced only to that precise spatial position on the proximal optical array end surface identifiable as fiber S85 at coordinates "4a". In this manner, only predetermined and pre-chosen fiber optical strands will receive light energy of determinable wavelengths for a specified duration; at a time desired by the user alone; and no other optical fiber strand will receive any light energy whatsoever other than those strands located at a precise spatial position on the surface of the optic array surface. By purposeful choosing, therefore, of which light sources on the lighting cable are to be employed, the user may introduce light energy at will to only pre-chosen, precise spatial positions and only to those few fiber optical strands known to be present at precisely that location alone on the optical array surface.

In most practical use instances, however, the lighting cable of Fig. 9 will not be employed because of its limitations. Recognizing that the typical cross-sectional diameter of a single fiber optical strand is only 2-20 micrometers; and recognizing further that the specie of oligonucleotide to be deposited precisely at a known spatial position on the optic array surface will desirably provide and encompass a surface area greater than the diameter of a single fiber strand; then clearly it is impractical and functionally unnecessary to employ only a lighting cable of such limited one-to-one correspondence as that shown by Fig.9.

In actual practice, therefore, a lighting apparatus having a pinhole in a filter holder which allows fine focusing and precise placement of light is employed in the making of the sensor. This pinhole apparatus has only one light source of illumination rather than a cable having multiple light sources; and the single pinhole acts as a light source to introduce focused light energy to several dozen individually clad, optical fiber strands simultaneously -- all the simultaneously illuminated strands being adjacently positioned within the imaging fiber optic array at precisely known spatial positions. In this manner, the single pinhole light source corresponds to and aligns with multiple fiber strands simultaneously; and permits the deposition of an oligonucleotide and monomer admixture over multiple strand faces simultaneously. The advantages and benefits of using the single source of focused lighting are that a controlled volume of admixture is precisely deposited at the pre-chosen spatial position on the optic array surface with minimal time and labor.

The lighting cable of Fig. 9, although completely operational for its intended

purpose, is often far too cumbersome for practical use; is provided only as a representative article to demonstrate the principle of introducing light energy to a precise location on the proximal optic array surface; and is used merely to illustrate the method and the manner in which the dye becomes photopolymerized and precisely positioned at a pre-chosen  
5 location on the distal optic array surface. Having illustrated both the principle and the intended results, it will be recognized and appreciated that any lighting source of any correspondence with the fiber optical strands of the fiber optic array will serve so long as the disposed oligonucleotide specie deposits are spatially separate and spatially distinguishable from one another on the optic array surface.

10

#### Photopolymerization

The manipulations performed during photopolymerization are illustrated via Figs. 10-15 respectively. For descriptive purposes only, the general magnified and oversimplified construction of the optic array surface of Fig. 7 and the lighting cable of  
15 Fig. 9 will again be used.

As seen within Figs. 10-15, a fiber optical connector 130 and illumination source 140 provide the capability for illuminating specific areas of one optic array surface of the imaging fiber optic array described previously. Thus, the light energy photons emanating from the surface of the illumination source 140 of Fig. 10 are produced by only light  
20 sources L23, L24 and L34 respectively. Only light energy at those precise spatial positions is directed towards the proximal optic array surface 114 of the unitary fiber optical array 100. Consequently, as shown via Fig. 10 only those fiber optical strands located at spatial position coordinates 10k, 10l, and 9k respectively receive the light energy photons provided by the illumination source 140. Then as illustrated by Fig. 11, only those  
25 corresponding individually clad fiber optical strands S23, S24 and S34 convey the introduced light energy through the body of the unitary fiber optical array 100; and the light exits at the distal optic array end surface 112 only at precise spatial positions (that is, solely at coordinate numbers XK, XL, and IXK as seen within Fig. 7 above. It will be recognized and appreciated that no other spatial positions on the distal array end surface  
30 112 are illuminated during this manipulation.

As the light energy photons emerge from the distal array end surface 112 at only the precise spatial positions indicated by Fig. 11, the optic array end surface 112 lies submerged in a prepared first monomer admixture. The light employed at only this precise

spatial positions was pre-chosen to be at a set wavelength for photopolymerization and the optic array end surface was allowed to react with the first monomer preparation for approximately 30 seconds duration. The reactive contact between the first monomer admixture and the light energy initiated a photopolymerization reaction on the distal array end surface and caused a deposition and an immobilization of the first polymer zone only at those illuminated spatial positions. Thus, at the end of the allotted reaction time for photopolymerization, a discrete volume 150 of a polymer was deposited and immobilized solely on the distal optic array surface solely at spatial positions XK, XIK, and IXK. No other fiber strands were illuminated; no other fiber strands conveyed any light energy whatsoever; and no polymerization or deposition occurred at any other spatial positions. This is illustrated by Fig.13.

After the first polymerization was completed, the illumination source 140 was then used again to illuminate the light positions corresponding to light position L15, L16, and L27. Light energy from only these positions introduced light energy photons precisely to the proximal optic array end surface 114 only at coordinate positions 10b, 10c, and 9c. This caused the introduced light energy photons to be conveyed solely by fibers SIS, S16 and S27. No other fiber strands were illuminated and no other fiber strands conveyed any light energy whatsoever. This is illustrated by Figs. 13 and 14.

Consequently, as appears in Fig. 14, light energy photons carried by only these individually clad, fiber optical strands (S15, S16, and S27) cause the light to be conveyed and to exit from the distal optic array end surface 112 only at coordinate position numbers XB, XC, and IXC. The optic array surface was then immersed in a prepared second monomer mixture and the light energy allowed to react with the prepared mixture for a predetermined duration. During this reaction time, photopolymerization proceeded and the second zone was deposited solely at those spatial positions which were illuminated. In this manner, the multiple deposits of different polymer zones became immobilized by photopolymerization at only those precisely illuminated locations identifiable by the coordinate numbers XB, XC and IXC. At the end of the allotted time for reactive contact, the distal optic array end surface of the imaging fiber optic array was removed from the second monomer admixture and revealed the deposition of an immobilized deposit of a second polymer matrix at the precise spatial positions identifiable precisely by coordinate numbers XB, XC and IXC. A discrete polymerized cone-shaped deposit 160 of the second oligonucleotide specie is seen extending from the distal optic array end surface as

illustrated by Fig. 15.

It will again be recognized and appreciated that under typical conditions the size of fiber core diameter in the fiber optic array so overwhelmingly exceeds the amount of corresponding cladding material that there is no effective separation between the fiber strands during the photopolymerization process. Thus the photopolymerization of the mixture at only the pre-chosen and illuminated spatial fluid positions results in the deposition of a single, unitary continuous volume large enough in surface area to encompass and cover multiple fiber end faces on the distal optic array surface. The presence of the cladding within the fiber optic array thus does not interfere with or hinder the continuity of the deposition. The result is both true and constant regardless of what specific process for depositing oligonucleotide species is employed and whether or not the favored photopolymerization technique is used.

The practitioner ordinarily skilled in this field will by now also recognize that there is no requirement or demand that an illumination fiber or cable as such be employed in this photoactivated method for making the sensor. To the contrary, one merely needs to introduce pinpoints of light into separate portions of areas of the imaging fiber optic array for photopolymerization to proceed. Thus, for example, one could achieve equivalent effects using lenses and/or lasers. Accordingly, any conventionally known means or manner of introducing light is deemed to be within the scope of the present invention.

The results of the completed photopolymerization process are illustrated by Fig. 15 in which the polymerized first oligonucleotide specie deposit 150 and the polymerized second oligonucleotide specie deposit 160 are individually located and identifiable at precise spatial positions on the distal optic array end surface. It will also be recognized that much of the distal optic array surface 112 remains unencumbered and unobscured; and that were additional light introduced at the proximal array end surface 114 at any of the unobscured strand spatial positions, such light photons would be conveyed and would exit from the distal optic array end surface 112 as unencumbered light energy which does not affect or influence the discrete deposits 150,160 positioned separately nearby.

### III. The Optical Sensing Apparatus And Instrumentation System

In order to be effectively employed, the prepared biosensor is combined with optical apparatus and instrumentation and is utilized as a system to detect and identify one or more specific analytes or peptide nucleic acids of interest. A generalized and



representative optical apparatus and instrumentation system which is conventionally available and preferably employed is illustrated by Fig. 16.

Sensor measurements may be performed using the apparatus shown schematically by Fig. 16 in the following manner: White light from an excitation source 200 (such as an arc lamp) is collimated; focused by a lens 201; is passed through an excitation filter 202; and is focused on an optic sensor 205 via a 10X microscope objective 204. The optic sensor 205 is held in an xyz-micropositioner 206 which allows for fine focusing. Excitation light is transmitted and illuminates each thin film sensing receptor unit in the array of the sensor which individually fluoresces in proportion to analyte concentration. The returning fluorescence light is reflected 90° by the dichroic filter 203; desirably, but optimally passed through a beam splitter cube 208; filtered at an appropriate emission wavelength by emission filter wheel 210; and then is detected by the CCD camera 220. Ratiometric measurements are obtained by monitoring fluorescence while switching between two excitation filters 202 using the emission filter wheel 210. The CCD camera typically contains a photosensitive element and may be coupled to an electronic intensifier; which in turn is connected to a computer having a Video Frame Grabber graphic card that digitizes and processes the video image. Visual imaging is achieved by using a CCD video camera to collect the light which is reflected 90° by the beam splitter cube. Illumination for visual imaging purposes is achieved either by rotating the excitation filter wheel to an empty position (using neutral density filters as necessary); or by illuminating the sample and its environs at the distal end of the sensor with an independent light source.

The optic sensing apparatus and instrumentation system shown by Fig. 16 detects fluorescence either as light intensity or as light wavelengths -- that is, a spectral response generated by and released by a deposited probe from a single optical fiber strand end surface; or from at least one strand end face in a bundled array of single core fibers; or from at least one individual spatial position on the distal array end surface of the unitary array after initial illumination with light energy of a pre-determined wavelength. The light energy emitted or reflected from each fixed probe position individually is collected using a CCD video camera using standard frame grabbing technology and image processing capabilities. Each spectral response detected as emerging light energy by the detector of the CCD is recorded; and the pattern of fluorescence or color is shown either as energy wavelength or as light intensity pixels on the detector representing the spatial dimension. By definition, a pixel is a picture element -- a sensitive region -- which determines light

intensity and/or light energy quantum.

#### IV. The Mobile Complementary Nucleic Acid Target Sequence

The analyte of interest to be detected optically using the biosensor and supporting apparatus is at least one mobile complementary oligonucleotide target specie bearing an identifying label joined directly or indirectly. The complementary target specie represents a sequence of nucleotides which corresponds as the complementary base sequence to the nucleic acids comprising the fixed oligonucleotide probe. It is expected and envisioned that the mobile complementary target specie will bind selectively to and hybridize in-situ with the nucleic acid sequence of the probe; and thereby generate a reaction product which is the result of specific and selective binding between only the fixed probe specie and the complementary target specie.

The present invention also intends and expects that an in-situ hybridization reaction will occur between each oligonucleotide specie deposit immobilized at one position or at multiple and differing spatial positions as fixed probes, each type of specie deposit reacting selectively with one mobile complementary oligonucleotide target specie (while other specie specific probes react concurrently with another alternative type of mobile complementary target specie). In this manner, different kinds of individually labeled complementary target species will react with only their counterpart and corresponding specie-specific fixed probes on at least one and preferably at several individual and differing spatial positions on the distal array end surface. Thus, at the user's choice and option, the array formats of the biosensor may be utilized to selectively detect only one mobile target species or a plurality of different target species intermixed in a fluid sample.

There are therefore only three requirements for each specie of complementary nucleic acid target to be detected using the present invention. These are: that the complementary target specie be mobile; that the complementary target specie ultimately bear a directly or indirectly joined identifying label; and that the complementary target specie be present in a fluid sample placed into reactive contact with the distal end of the biosensor. It will be noted and appreciated also that while there is no requirement as such, it is often desirable that the individual complementary oligonucleotide target specie be separated, isolated, purified or semi-purified before being placed into reactive contact in order for an effective in-situ hybridization to occur. Thus, the fluid sample often may comprise a mixture of different materials including not only multiple nucleic acid

sequenced fragments, some of which are the complementary target specie for hybridization; but also may contain some extraneous matter, such as cellular debris or unrelated pharmacologically active molecules which typically are present only as incidental remnants from an earlier extraction, reaction, or preparation process. As a  
5 general rule, therefore, the fewer the number of extraneous chemical entities in the fluid sample, the faster the kinetics of in-situ hybridization will proceed.

The light energy absorbing dyes useful as joined identifying labels

At least one light energy absorbing dye is bound initially or becomes linked  
10 subsequently to each mobile complementary oligonucleotide target species as an identifying label. If desired, more than one dye reagent can be employed as a joined identifying label.

Each light energy absorbing dye formulation or composition will be bound directly or becomes linked indirectly to the one specie or to multiple different species of  
15 oligonucleotides intended for use as complementary targets. Moreover, each dye will then show evidence of its presence by either absorbing and reflecting a portion of the light energy; or, alternatively, by absorbing light energy and then subsequently emitting light energy of a different wavelength in return. Such reflected or emitted light energy is intended to be conveyed from the distal end surface; and such conveyed light will emerge  
20 from the proximal end surface for detection and measurement.

The various dyes which may be bound initially or linked subsequently to a chosen oligonucleotide fragment as a joined identifying label are all conventionally known and often commercially available. The present invention intends that all the commonly useful properties and capabilities of the various classes of light energy absorbing dyes be  
25 employed directly and indirectly, and as needed or desired for the specific use or application. Merely illustrative of the many different dyes are those fluorophores, indirect (secondary) labels, and interchelators listed below within Tables 2, 3, and 4 respectively.

Table 2

<u>Compounds</u>	Excitation Wavelength (range or <u>maximum</u> )	Fluorescence emission range ( <u>max</u> )
A. <u>Fluorophores</u>		
Eosin	520-530 nm	530-580 nm (550 nm)
TRITC-amine	555 nm	570-619 nm (590 nm)
Quinine	320-352 nm	381 -450 nm
Fluorescein W	488-496 nm	530 nm
Acridine yellow	464 nm	500 nm
Lissamine Rhodamine	567 nm	580 nm
B Sulfonyl Chloride		
Erythroscein	504 nm	560 nm
Ruthenium (tris, bipyridium)	460 nm	580 nm
Texas Red	591 nm	612 nm
Sulfonyl Chloride		
B-phycoerythin	545, 565 nm	575 nm
Nicotinamide adenine	340 nm	435 nm
dinucleotide (NADH)		
Flavin adenine	450 nm	530 nm
dinucleotide (FAD)		
Carboxy	587 nm	640 nm
Seminaphthorhydafluor		
Naphthofluorescein	594 nm	663 nm
Carboxy Fluorescein (Fam)	495 nm	520 nm
BODIPY*		
JOE*		
TAMRA*	540 nm	564 nm

ROX*	567 nm	591 nm
B. <u>Fluorescent Antibody Conjugates</u>		
Protein A fluorescein conjugates	480 nm	520 nm
Anti-Atrazine fluorescein Conjugates	480 nm	520 nm
digoxin-Anti-digoxin Texas Red Conjugates	590 nm	615 nm

\* Trademarks of Molecular Probes Inc. (Eugene, OR)

Table 3:

## Secondary Label Pairs

(Labels include but are not limited to those mentioned in Table 2)

Biotin	Labeled avidin / streptavidin
Protein A	Labeled IgG
Digoxin	Labeled anti-digoxin
Enzymes such as:	
alkaline phosphatase	diphosphate derivatives
	ELF-97 substrates (enzyme activated)
$\beta$ -glucuronidase	glucuronide
	galactopyranoside
Horseradish Peroxidase	

Table 4:Intercalators

5

ethidium bromide;

Cy-5;

10

Ru (byp)<sub>2</sub> MCCP;

Hoechst 33258 (bis-benzimide);

Cyanine dyes\*

15

TOTO®

YOYO®

BOBO™

POPO™

SYBR I®

20

\* Trademarks of Molecular Probes Inc. (Eugene, OR)

It will be recognized and appreciated also that the available range, variety, and diversity of light energy absorbing dyes, dye formulations, and dye mixtures is not dependent upon a single light source or light energy supply in order to be effective. Although light energy of determinable wavelengths is desirably provided by electrical light sources -- that is, light emitting diodes (LEDs), lasers, laser diodes and filament lamps whose bands of light energy are typically controlled and selected by filters, diffraction gratings, polarized filters; or alternatively broken into various broad wavelengths of light energy via prisms, lenses, or other optical/spectral articles, these are not exclusively the only source of useful light energy. Clearly, in various applications and circumstances other less typical light energy sources will also be useful. Accordingly, neither the true source, nor the nature of light energy photons, nor the manner in which they are conveyed or otherwise caused to be created is of importance or consequence.

In addition, the dye label individually may comprise a pair of specifically binding materials such as the chemical compounds listed within Table 3 for subsequent reactive contact and indirect juncture of an identifying label. Thus each dye label individually may in fact be formulated as a composite comprising light emitting dye in part; and include a variety of receiving elements which are able to interact as specific binding partners for joining the light energy dye label subsequently. Exemplifying some multiple formulations and combinations are those described below and used experimentally hereinafter.

#### Methods for preparing a mobile complementary target specie bearing an identifying label

A range of different preparation methods and processes are conventionally known and available in the published scientific literature for creating a mobile complementary oligonucleotide target specie of known base sequence joined directly to an identifying label as a conjugate. Representative of and exemplifying these direct attachment procedures are the following: Agrawal et al., Nucleic Acids Res. 14: 6227-6245 (1986); Smith et al., Nucleic Acids Res. 13: 2399-2412 (1985); Cardullo et al., Proc. Natl. Acad. Sci. USA 85: 8790-8794 (1988); J Fluorescence 1:135 (1991).

An alternative preparation strategy and procedure is exemplified by incorporation of biotin, in the form of a biotinylated nucleotide (such as biotin-d UTP) into the nucleic acid structure using conventional procedures such as nick translation or tailing [Rigby et al., J. Mol. Biol. 113: 237 (1977); Lobban, P.E. and A.D. Kiser, J. Mol. Biol. 78: 453 (1973)]. The selective binding of the biotinylated targeted to the fixed probes in the in-situ



hybridization reaction may then proceed in the absence of the dye label itself.

Subsequently a conjugate complex constituted of avidin or streptavidin (proteins with a high affinity for binding to biotin) covalently are linked to a fluorescent or color reflecting dye ligand is then added as a dye label complex to the reaction fluid after hybridization has occurred; and the selective binding capability for the paired agents will then cause the identifying dye label to be joined via the avidin (or streptavidin) indirectly to each biotinylated target species wherever it is found. Qualitative and quantitative optical detection of the hybridized target specie can therefore be made on the basis of the spectral characteristics of the ultimately and indirectly joined identifying label. The listing of Table 3 provides other alternative pairs of specific binding agents suitable for use.

If desired, a biotinylated complementary target specie may be also prepared using polymerase chain reaction processing. The biotinylated amplified product obtained by PCR methodology may be used immediately or purified before being placed into reactive contact with the biosensor. Also, any of the other pairs listed in Table 3 may be substituted for use in the PCR method.

In addition, the user may optionally employ the technology known as enzyme-labeled fluorescence (ELF) signal amplification to provide a joined identifying label. This technology is described in detail by U.S. Patent Nos. 5,136,906 and 5,443, 986, the texts of which are expressly incorporated by reference herein. In brief, an enzyme such as alkaline phosphatase is attached directly or is linked subsequently to the complementary oligonucleotide target species. The substrate for the enzyme's catalytic activity is one which provides an intense fluorescent signal and also demonstrates a very large Stoke's shift. Such substrates have been shown to be highly detectable labels when used with conventional in-situ hybridization method. See for example: Am.J. Human Genet. Suppl. 55, A271, Abstract #1588 (1994); FASEB J. 8: A1444, Abstract #1081 (1994); and Mol. Biol. of the Cell Suppl. 4, 226a, Abstract #1313 (1993).

The user is thus given the option of preparing the complementary oligonucleotide target specie in several ways. The target specie nucleic acid sequence may be directly and immediately bound to an identifying dye label (such as those of Table 2) if desired. Alternatively, the complementary oligonucleotide target specie or species may be prepared as molecules having a receiving element such as biotin. The biotinylated target specie is allowed to hybridize in-situ with the fixed probes on the distal end surface of the biosensor; and then a prepared specific binding partner (such as avidin or streptavidin

complex) bearing an identifying dye label as a component part can then be added to the reaction fluid after hybridization is completed -- thereby causing the identifying label to be joined subsequently as well as indirectly to the immobilized hybridized reaction product. Finally, the intercalators (such as those listed within Table 4), may be used in unmodified form to label double-stranded, hybridized reaction products without any prior intermediate agent. The target species thus remains unlabeled throughout the entire hybridization process; the intercalators then will bind directly to the double-stranded reaction product upon reactive contact.

10 V. Identifying The Resulting, Species-Specific, In-Situ Hybridization  
Oligonucleotide Reaction Product

The optical biosensor of the present invention intends and requires that a species-specific, in-situ hybridized reaction product result as the consequence of a selective reactive contact between a mobile complementary oligonucleotide target specie; a subsequently linked or directly joined identifying dye label; and one or more specie selective oligonucleotide probes deployed either on the distal end surface of a single optical fiber strand or at differing fixed spatial positions upon one array end surface of a unitary fiber optic array. Two features are essential for the biosensor: (a) The individual spatial positioning of the individual specie deposits as multiple fixed probes deployed on the distal end surface of the biosensor allows for a single or many different and distinct specie-specific, in-situ hybridizations to occur with a minimal volume of a mobile complementary oligonucleotide target specie having an initially existing or subsequently joined identifying label; and (b) the predetermined and fixed, individual spatial positioning(s) of the deployed, single specie, fixed probe(s) upon the discrete optic end surface utilize the initially or ultimately joined identifying label to identify the occurrence of a species-specific hybridization in-situ. Also each biosensor format relies upon spatial resolution as a means by which to differentiate and distinguish among the alternatively positioned hybridized complementary target species concurrently immobilized at many differing spatial locations on the optic array surface. In the fiber optic array format and in the bundle of fibers format, therefore, it is the combination of fixed spatial positionings for in-situ hybridization at the differing chosen locations and the spatial resolution capability to separate and distinguish the joined identifying dye label among the differing fixed surface positionings (and the different, hybridization reactions occurring concurrently)

which avoids and eliminates random intermixing of individual light energy photons traveling to and from an identifying label concomitantly joined to each in-situ hybridized reaction product formed on the optic array surface of the biosensor.

Thus, within the in-situ hybridization zone on the distal end surface, each  
5 identifying label directly or indirectly joined to a hybridized complementary  
oligonucleotide target specie becomes, in turn, concomitantly immobilized and disposed  
only at individual probe locations fixed on the optic array end surface and the presence of a  
joined identifying label held at any one or more fixed spatial positions can only be the  
secondary consequence of a specie-specific in-situ hybridization reaction having occurred  
10 at that spatial position. Each immobilized identifying dye label will then show evidence of  
its presence at that precise spatial position by either absorbing and reflecting a portion of  
the light energy or absorbing light and then subsequently emitting light energy of a  
different wavelength in return. Such reflected or emitted light energy is conveyed via one  
or more individual fiber optic strands in aligned position with the immobilized dye itself.  
15 Such conveyed light will emerge from the other optic end surface only at precisely located  
spatial positions; and thus be distinguishable as such from other light energy conveyed by  
any other fiber optical strands via the precise spatial positioning and the spatial resolution  
of the emerging light at the optic array surface. In this manner, the conventional limitations  
and demands of single channel optical fibers are eliminated since the strands within the  
20 fiber optical array retain the spatial positioning for each of the disposed dye labels. Thus,  
the traditional requirement for spectral resolution is removed due to the ability by the fiber  
optical array to resolve each of the dye labels spatially.

25

## VI. Experiments And Empirical Data

### Experimental Series A

#### Single optical fiber strand preparation

The distal and proximal faccs of several single optical fiber strands are polished  
30 and cleaned. Each strand's distal end was silanized in 10% (amino) propyltriethoxysilane  
in acetone (v/v). The single core fiber strands were removed after 2 hours, rinsed with  
acetone, and then air dried for 30 minutes. The single core strands were placed in a 1.25%  
gluteraldehyde solution in 0.02 M phosphate buffer (pH 6.8) for 30 minutes. The single

core fiber strands then were rinsed with distilled water and placed in 3% polyethyleneimine (PEI) in 0.02 M phosphate buffer (pH 6.8). Finally, the single core strands were air dried for 1 hour, rinsed, and stored in distilled water until being functionalized individually with oligonucleotides.

5

#### Bundled optical fiber array preparation

To make a bundled optical fiber array, seven functionalized 200 $\mu$ m diameter single core fiber strands were bundled together. The distal end surface of each fiber strand was first functionalized as described herein with a different cytokine oligonucleotide as a fixed probe - the bundling of seven individual strands creating a multi-target sensing array. The proximal ends of the bundled optical fiber array were epoxied into a 1 mm stainless steel tube (A) and placed into the fiber chuck of the epifluorescence imaging system as shown in Fig. 17. The bundled optical fiber array was three feet long in length and was used for remote sensing (B). The protective rubber tubing is removed from the distal end of the bundled arrays to enable individual functionalization (C).

15

#### Oligonucleotide preparation:

10 nmoles of 5'-amino-terminal oligonucleotide were dissolved in 90  $\mu$ L of 0.1 sodium borate buffer (SBB). Oligonucleotide activation was initiated by adding 5 nmoles of cyanuric chloride in 10  $\mu$ L of acetonitrile. The reaction proceeded at room temperature for 1 hour. Unreacted cyanuric chloride was removed by three cycles of centrifugal ultrafiltration (3000 d MW cutoff, Microcon 3, Amicon) and diluted with 200  $\mu$ L of 0.1 M SBB. The activated primers were recovered in approximately 50  $\mu$ L of 0.1 M SBB and stored at 4°C. Primers were used within one month of activation.

25

#### Single core fiber sensor preparation:

The modified distal end of each single core strand was washed by dipping several times in five-2 ml changes of 0.1 M SBB. The distal strand tips were immersed in a 10  $\mu$ L solution of 150  $\mu$ L cyanuric chloride-activated oligonucleotide in 0.1 M SBB for 1-2 hours. The fiber strand tips were then immersed in 200  $\mu$ L of 90% DMSO, 10% 1 M SBB buffer pH 8.3, 0.1 M succinic anhydride for 1 hour at room temperature. The fiber tips were washed (as described above) with 2 changes of 0.1 M SBB, 5 changes of TE (10 mM Tris-HCl pH 8.3, 1mM EDTA) containing 0.1 M NaCl and 0.1% SDS. Fibers tips were

30

stored in the washing buffer until use.

Fluorescence measurements:

Fluorescence measurements were acquired with a modified Olympus  
5 epifluorescence microscope / charged coupled device camera described previously herein.

Primary PCR reaction:

A 176 base pair region from a human IL-4 cDNA plasmid clone [Yokota et al.,  
Proc. Natl. Acad. Sci. USA 83: 5894-5898 (1986)] was amplified using primers IL4-U (5'-  
10 CATCGTTAGCTTCTCCTGA-3') and IL4-L (5'-AAAGTTTTGATGATCTCCTGTA-3')  
generating a double-stranded PCR product. Conditions for the reaction were 10 mM Tris-  
HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.5% Tween-20, each primer at 0.5 μM dNTPs,  
2 U AmpliTaq polymerase, 10<sup>4</sup> copies of BamHI-cleaved pcd-hIL-4. Thermocycling  
parameters were 35 cycles consisting of 10 seconds at 94°C, 10 seconds at 55°C, and 30  
15 seconds at 72°C.

Secondary PCR reaction:

The double-stranded primary amplification products were internally labeled during  
an asymmetric PCR step using fluorescein-labeled dGTP. Conditions for the reaction were  
20 10 mM Tris-HCl pH 8.3; 50 mM KCl; 2.5 mM MgCl<sub>2</sub> 0.5% Tween-20;  
1 μM unlabeled primer IL-4 L; 50 μM each dATP, dCTP, dTTP; 25 μM dGTP; 25 μM  
dGTP; 25 μM fluorescein-labeled dGTP. After asymmetric amplification, the reaction was  
extracted with phenol and isobutanol. Primers and nucleotides were removed by two cycles  
of centrifugal ultrafiltration (Microcon 30, Amicon 400 μL TE per wash). Samples were  
25 recovered from the filtration unit in 50 μL of TE.

PCR Product Assay

The array distal end was placed in 5 μL of 16 nM labeled PCR product in buffer  
(TE containing 0.1% SDS and 0.35 M NaCl) for 20 minutes then rinsed with buffer  
30 solution (TE containing 0.1 % SDS and 0.35 M NaCl). Signal was acquired while the  
biosensor array was in buffer solution.

Experiment 1: The Biosensor

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To create a DNA biosensor array, probes specific for human cytokine mRNA sequences were immobilized on the tips of single core optical fibers. Cytokines are powerful immune system hormones whose expression is stimulated in response to inflammatory stimuli or infection. For this reason, there is widespread interest in the regulation of the cytokine gene expression. The sequences of the cytokine probe and targets used are shown in Table EI.

To make a bundled optical fiber array, seven functionalized 200  $\mu$ m diameter single core fibers were combined and bundled together using epoxy. The distal end surface of each optical fiber strand was first functionalized as described above with a different cytokine oligonucleotide as a fixed probe; and then the bundling of seven functionalized strands created a multi-target sensing array. The proximal ends of the bundled optical fiber array were then epoxied into a 1 mm stainless steel tube and placed into the fiber chuck (A) of the epifluorescence imaging system as shown in Fig. 17. The bundled array was three feet long in length and was used for remote sensing (B). The protective rubber tubing is removed from the distal end of the bundled array to enable individual functionalization (C).

The bundled array of optical fibers was then tested by placing the distal end of the bundled strand sensor into a solution containing one or more 5'-fluorescein-labeled cytokine sequences. The bundled array was removed from the target solution after incubation for 5 minutes and rinsed with buffer solution (TE, 0.1 % SDS, 0.1 M NaCl). The bundled array was then placed in buffer solution and fluorescence signals were acquired (excitation wavelength 490 nm emission wavelength 530 nm). Signal analysis was performed in less than 30 seconds with commercially available software (IP Lab Spectrum). The results are shown by Fig. 22.

The images of the bundled array biosensor in buffer solution as acquired by a CCD camera are shown by Figs. 18A and 18B. The image with white light transmitted through the distal end of the array is demonstrated by Fig. 18A. In comparison, a background fluorescence image at 530 nm taken with 490 nm excitation in buffer solution is shown by Fig. 18B. While there is some light transmitted through the cladding, there is no fluorescence observed across the distal end surface of the bundled array.

Table E1

Probe Sequences	Target Sequences
$\beta$ -glo(+)(segment of human $\beta$ -globin)	$\beta$ -glo(+)-CF
5'-(NH <sub>2</sub> -(CH <sub>2</sub> ) <sub>6</sub> )DTT TTT TCA ACT TCA TCC ACG TTC ADD-3'	5'-Fluorescein-TG AAC GTG GAT GAS GTT G-3'
IFNG (interferon gamma 1)	IFNG-CF
5'-(NH <sub>2</sub> -(CH <sub>2</sub> ) <sub>6</sub> )T12-TGG FTT CTC TGG GCT GTT ACT-3'	5'-Fluorescein-AG TAA CAG CCA AGA GAA CCC AAA-3'
IL2 (interleukin-2)	IL2-CF
5'-(NH <sub>2</sub> -(CH <sub>2</sub> ) <sub>6</sub> )T12-TA CAA GAA TCC CAA ACT CAC CAG-3'	5'-Fluorescein-CT GGT GAG TTT GGG ATT CTT GTA-3'
IL4 (interleukin-4)	IL4-CF
5'-(NH <sub>2</sub> -(CH <sub>2</sub> ) <sub>6</sub> )T12-CC AAC TGC TTC CCC CTC TGT-3'	5'-Fluorescein-AC AGA GGG GGA AGC AGT TGG-3'
IL6 (interleukin-6)	IL6-CF
5'-(NH <sub>2</sub> -(CH <sub>2</sub> ) <sub>6</sub> )T12-GT TGG GTC AGG GGT GGT TAT T-3'	5'-Fluorescein-AA TAA CCA CCC CTG ACC CAA C-3'

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Experiment 2:

Background-subtracted mean fluorescence intensities were then obtained with IL-4 probe on the sensor. The bundled strand biosensor array was tested in different target solutions employing the sequences of probes and targets shown in Table EI. The biosensor  
5 was placed in a target solution for 5 minutes; rinsed with buffer solution (TE containing 0.1% SDS and 0.1 mM NaCl); and fluorescent images were then acquired with the fiber tip in buffer. The result is shown by Fig. 19.

The bundled strand array was dipped in 90% formamide in TE between each test to remove any hybridized target and to regenerate the sensor. The IL-4 target solution contained  
10 fluorescein labeled oligonucleotide complementary to the IL-4 probe on the sensor. After hybridization and read-out, the sensor was regenerated and used to test the IL-2 and IFNG-target solutions containing fluorescein labeled targets that are not complementary to the IL-4 probe. As seen in Fig. 19, these targets do not hybridize to the sensor. The second IL-4 hybridization afforded a signal close to the original test.

15 Plots of background subtracted mean fluorescence at 530 nm were then taken with 490 nm excitation as a function of time using a 500  $\mu$ m diameter single core sensor with  $\beta$ -glo probe. A kinetic study using 1  $\mu$ M  $\beta$ -glo target solution is shown by Fig. 20. Also, a kinetic study using 0.1  $\mu$ M  $\beta$ -glo target solution is shown by Fig. 21. The sensor was placed in each  
20 of the  $\beta$ -glo target solutions for a given time; rinsed with buffer solution (TE containing 0.1 % SDS and 0.1 M NaCl); and a fluorescence signal was acquired with the sensor in buffer solution. After data acquisition, the fiber optic array biosensor was placed back in the target solution for an allotted time, rinsed, and examined in buffer. Once the kinetic data was acquired, the hybridized target was removed with 90% formamide in TE to regenerate the sensor.

25 Fluorescent images using the fiber optic biosensor array were acquired in buffer solution after treatment with IL2 target (A); IL4 target (B); IL6 target (C);  $\beta$ -glo target (D); IFNF target (E); and a mixture of IL4, IFNG and  $\beta$ -glo targets. The biosensor array distal end was placed sequentially in a fluid (A)-(F) using a 1  $\mu$ M solution in TE containing 0.1% SDS and 0.1 M NaCl for 5 minutes; and was then rinsed with buffer solution (TE containing 0.1%



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SDS and 0.1 M NaCl). Hybridized oligonucleotides were removed after each immersion and optical detection using a 90% formamide solution in TE after each analysis. As shown by Fig. 22, the specificity of each probe-target hybridization (A)-(F) was confirmed. The signals obtained in buffer before hybridization were subtracted from the signals obtained after hybridization. High intensities are signified with white light.

The background subtracted mean fluorescence signals from Fig. 22 are shown in graph form by Fig. 23. Note: When a combination of oligonucleotide targets was placed in solution, the signal obtained was comparable to the signal obtained when a single target was in the solution. Figs. 22 and 23 in combination reveal that high signals were only observed on those fibers carrying probes complementary to the added target, demonstrating that hybridization to the fiber is also highly specific.

#### Experiment 4:

Hybridization competition between labeled and unlabeled variations of the same target sequences were performed using the biosensor array. Increasing concentrations of unlabeled targets were added to 1- $\mu$ M solutions of a labeled identical target. The optical fiber biosensor was placed in the mixture of labeled and unlabeled target solution for 10 minutes; rinsed with buffer solution; and the background-subtracted fluorescence image was acquired while the distal array tip was in the buffer solution. The distal tip was rinsed with 90% formamide in TE between test samples to remove the hybridized target. The results are shown by Fig. 24; the fluorescence decrease was directly proportional to increasing concentration of sample target.

#### Experiment 5:

A practical application of the fiber optic biosensor is shown by the assay of cDNA samples generated by reverse-transcription PCR (RT-PCR) [Egger, et al J Clin. Microbiol. 33: 142 (1995)]. A 176 base pair fragment containing the IL-4 probe sequence (Table EI) was amplified from a cloned IL-4 cDNA target, and internally labeled with fluorescein using an asymmetrical PCR procedure [Cronin et al., Human Mutation 7: 244-255 (1996)]. The cytokine sensor array was dipped directly into the purified PCR reaction mixture. Using 5  $\mu$ L of a 16 nM PCR target solution the sensor gave a clear, detectable, specific signal after 20

-48-

minutes.

#### Optimization of the system

Immobilized probe concentrations limit the amount of complementary target that can  
5 hybridize to the probe and generate a signal. The feasibility of detecting a fluorescein labeled  
target in-situ hybridization to the immobilized probe on the distal surface was tested first  
using a 500  $\mu\text{m}$  diameter single core fiber with an immobilized IL-4 probe (Table E-1). The  
tips of the array (surface area  $0.002\text{ cm}^2$ ) were placed in the target solution, which can be as  
small as 3  $\mu\text{L}$  in a 0.4 mL eppendorf tube. The detection system consists of a modified  
10 epifluorescence microscope with the optics optimized to couple with an optical fiber.

The detection system parameters were also optimized. Increasing the acquisition time  
improved the signal however, the increased exposure time caused photobleaching and  
adversely affected subsequent sensor use. A two second acquisition time was found to be  
optimal in maximizing the detection while minimizing photobleaching of the fluorescein-  
15 labeled target. This fiber optic biosensor system has a detection limit of 10 nM.

#### Characterization of the sensor

The sensor's specificity was evaluated by placing the sensor in complementary and  
non-complementary-labeled target solutions. The fluorescent signal increases upon exposure  
20 to the complementary labeled target. The complete absence of signal when the biosensor was  
placed in a non-complementary labeled targets confirmed the hybridization specificity (Fig.  
19).

Lengthy incubation times are frequently a concern in hybridization experiments  
utilizing immobilized probes. The optical fiber biosensor described here shows excellent  
25 hybridization kinetics. Hybridization is 85% complete after 1 minute using a 1- $\mu\text{M}$  target  
solution (Fig. 20). With a 0.1- $\mu\text{M}$  target solution, hybridization is 90% complete after 15  
minutes (Fig. 21). The 10-nM target solution required 10 minutes to generate a clear signal  
(data not shown).

Fiber biosensors can be regenerated repeatedly by dipping the distal array tip in 90%  
30 formamide in TE buffer (10mM Tris-HCl pH 8.3, 1mM EDTA) for 10 seconds at room

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temperature. The biosensor then gives an original comparable signal for all subsequent analyses with alternative complementary oligonucleotide target (Fig. 19). Heating the formamide solution to 45°C was also effective and did not compromise sensor integrity.

The biosensor offers significant advantages for hybridization analysis. The optical fiber array serves as the hybridization support and also facilitates sensitive, quantitative fluorescent detection of in-situ hybridization using a very low sample volume. Multiple synthetic oligonucleotide probes were covalently immobilized and fixed on one end of a 200 µm diameter optical fiber.

The fiber optic biosensor and methodology demonstrates a fast reproducible, highly sensitive and durable system for the specific identification of DNA sequences. Fiber optic biosensor arrays enable simultaneous detection of multiple DNA sequences with reduced assay time and increased convenience. Complete analysis of multiple DNA sequences can be accomplished in under 5 minutes. Biosensors can be prepared in advance and be stored at 40°C; these maintain their sensing capabilities for many months. The biosensor's small size also offers the ability to perform hybridization analysis on large numbers of target sequences using extremely small sample volumes. The biosensor is also useful for performing in-situ hybridization analyses in settings where conventional hybridization methods would be difficult, if not impossible, to implement.

#### Experimental Series B

##### Experimental Protocol:

The approach to DNA immobilization involves the site-selective photodeposition of an acrylamide and N-acryloxysuccinimide copolymer. The distal face of a fiber optic imaging array is first functionalized with 3-trimethoxysilylpropylmethacrylate to attach the photopolymerizable acrylate to the glass and surface allowing for the covalent attachment of polymer matrices to the distal face. The proximal array end surface of the unitary array is placed on a photodeposition system which allows for site-positioned illumination of the proximal array end. The distal end of the functionalized array is then dip coated with a thin film of an acrylamide/acryloxysuccinimide prepolymer containing a photoinitiator. The prepolymer is then photopolymerized by light illumination through the fiber for a fixed time

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and excess prepolymer is removed by rinsing with ethanol. After the polymer matrix is deposited, the distal end is placed in a solution of a 5'-amino-terminated oligonucleotide. As the polymer then hydrates, the oligonucleotide reacts with the succinimidyl ester residue, thereby covalently immobilizing the oligonucleotide. The residual reactivity of the esters is capped by placing the distal end in a 1 mM ethanolamine buffer solution, pH 8.5. The process is subsequently repeated to immobilize other oligonucleotide probes. Once fabricated, the DNA sensor array is connected to a modified epifluorescence microscope (Olympus) with computer-controlled excitation and emission filter wheels and a frame-transfer charge coupled device (CCD) camera (Photometrics) as previously described herein.

10

#### Experiment 6

Initial studies were performed using a poly (dA) sensor array comprised of both an immobilized poly (dA) polymer matrix and a control acrylamide polymer matrix, which was not copolymerized with N-acryloxysuccinimide, using a poly (dT)-FITC target. The biosensor was placed directly in a dilute solution of poly (dT)-FITC. During hybridization of poly (dT)-FITC to the poly (dA) polymer matrix, poly-FITC concentrates in the polymer matrix resulting in an increase in fluorescence over the background solution fluorescence. The acrylamide matrix thus serves as a control for non-specific absorption of target. After hybridization to the poly (dT)-FITC, the poly (dA) biosensor array could be regenerated by immersing in 65°C buffer for 15 minutes after hybridization. This procedure completely dehybridized the probe/target duplex. Table E2 lists the oligonucleotide probes used in this study along with the complementary target sequences.

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Table E2:

Probe Name (code)	Sequence 5'-3'	Sequence 5'-3'	Target Name (code)
(p(dA))	H2N-(a) <sub>18</sub>	FITC-(T) <sub>18</sub> Biotin-(T) <sub>18</sub>	(p(dT)-FITC) (p(dT)-biotin)
H-ras wild type (H-ras Wt.)	H2N- CCGGCGGTGT	FITC- ACACCGCCGG	Wild type target (Wt-FITC target)
H-ras mutant (H-ras Δ)	H2N- GCCGTCGGTGT	FITC- ACACCGACGGC	Mutant target (Δ-FITC target)
		5'-labeled biotin amplicon containing ACACCGACGGC	PRC amplicon Δ PCR 109 bp

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Fig. 25 shows the fluorescence increase of the poly (dA) matrix upon repeat hybridizations to poly (dT)-FITC; and shows three repeat hybridizations after regenerating the poly (dA) sensor array with 65°C buffer. The signal increases are essentially the same for all three hybridizations when the decrease in solution fluorescence is taken into account as measured with the acrylamide matrix control. The plot demonstrates that sensor regeneration occurs in less than 10 seconds, simplifying and speeding up the testing procedure.

The poly (dA) biosensor array was then tested for its time response to various concentrations of poly (dT)-FITC. This data is shown by Fig. 26. The sharp decrease between standards represents the biosensor regeneration with 65°C buffer.

Other determinations were then made. Fig. 27 plots the initial hybridization rate versus poly (dT)-FITC concentration. The poly (dA) sensor array shows a linear response from 1.3-130 nM with an  $R^2$  value of 0.99 demonstrating that the rate of hybridization is directly proportional to the concentration of the target oligonucleotide. This linearity indicates that the response time of the DNA sensor array is diffusion controlled; and that the oligonucleotide probes are immobilized on the surface of the polymer matrix; allowing for solution-type kinetics.

#### Experiment 7:

A different labeling procedure was developed to increase the array's sensitivity and expand its generality. Presently PCR samples for fluorescence analysis must be labeled after amplification. However, PCR samples can be biotinylated during the amplification which decreases the time to analysis and minimizes sample contamination. The detection procedure involves hybridization with a biotinylated nucleic acid target followed by label juncture with streptavidin-FITC.

Accordingly, calibration of the poly (dA) matrix with poly (dT)-Biotin; and hybridization was carried out for 20 minutes at 0.2, 2.0 and 19.6 nM followed by development with streptavidin-FITC. The results are shown by Fig. 28 which plots the mean fluorescence intensity of the poly (dA) matrix versus the log of poly (dT)-biotin concentration. As in a diffusion controlled system, the fluorescence intensity is shown to be linear with the log poly (dT)-biotin concentration from 0.2-20 nM. This system has a detection limit approximately an

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order of magnitude lower in target concentration for the same in-situ hybridization time. This improvement is due to the multiple FITC labels on each streptavidin molecule and the absence of photobleaching during hybridization that otherwise occurs with FITC-labeled oligonucleotides. The technique has a detection limit of 0.2 nM which is approximately an  
5 order of magnitude lower than other DNA biosensors.

#### Experiment 8:

In order to test if the DNA biosensor could distinguish single point mutations, an array comprised of multiple H-ras wild type probes (H-ras Wt.) and multiple mutant probes with a  
10 single base difference (H-ras  $\Delta$ ) was fabricated. A FITC-labeled mutant ( $\Delta$ -FITC) target was hybridized to the DNA sensor array at 28°C in low stringency buffer, 2X SSPE. The  $\Delta$ -FITC target is the perfect complement for the immobilized H-ras  $\Delta$ . The low stringency buffer condition was insufficient to distinguish the non-complementary oligonucleotide target at 28°C.

15 As temperature is often utilized to distinguish non-complementary targets, thermal studies were then undertaken to determine the melt characteristics of the duplexes. The distal array end of the biosensor was immersed in buffer solution and the temperature was raised while monitoring fluorescence. Fig. 29 shows the melting curves of the DNA sensor array after hybridization at 28°C with the  $\Delta$ -FITC target. The plot shows that  $T_m$  for the H-ras  
20 Wt./ $\Delta$ -FITC target duplex occurs at approximately 42°C while the  $T_m$  for the perfect complement duplex H-ras/ $\Delta$ -FITC target occurs at 55°C. The data shows that if hybridization is performed at approximately 54°C, only the complementary target will hybridize.

Figs. 30A and 30B respectively show fluorescence images of the DNA sensor array after hybridization with the  $\Delta$ -FITC target at 28°C and 54°C, A and B respectively. The  
25 images indicate that the DNA sensor array can distinguish a point mutation when hybridization is conducted at 54°C. Images were acquired at 490 nm.

The DNA sensor array was then calibrated with the  $\Delta$ -FITC target by the same procedure used for the poly (dA) sensor array and poly (dT)-FITC with the exception that hybridizations were carried out at 54°C. Fig. 31 shows hybridization data for the DNA sensor

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array to 196 nM  $\Delta$ -FITC target at 54°C. The H-ras Wt. matrix showed no response to the H-ras  $\Delta$ -FITC target. Fig. 32 is a plot of the  $\Delta$  target calibration curve for the data of Fig. 31 which demonstrates the sensitivity of the sensor in the concentration range 20 196 nM.

Experiment 9:

5 In order to test the array's ability to distinguish point mutations of amplified DNA, a PCR sample containing the A target sequence was obtained. The target sample, biotinylated during PCR amplification, was first determined and diluted; allowed to hybridize to the DNA sensor array for 20 minutes at 54°C; washed and then labeled by juncture to streptavidin-FITC.

10 Fig. 33 shows a fluorescence image of the DNA sensor array after a 20 minute hybridization to a biotinylated  $\Delta$  PCR amplification at 54°C, followed by a 5 minute streptavidin-FITC development. The image was acquired at 490 nm excitation, 530 nm emission. The image of Fig. 33 demonstrates that the biosensor can distinguish single base mutations in amplified DNA.

15 A fiber optic DNA sensor array has been fabricated by photodeposition of amine-reactive polymer matrices on an imaging fiber optic array; and 5'-aminoterminal oligonucleotides have been covalently immobilized as multiple fixed probes through amide bond formation with the succinimidyl ester residues of the polymer matrices. This fiber optic DNA sensor array is capable of simultaneously monitoring multiple hybridization events. The  
20 DNA sensor array also has the added advantage of simultaneously evaluating a fluid mixture of target oligonucleotides with multiple kinds of probe oligonucleotides with real-time monitoring. The DNA sensor array can be utilized to identify a single point mutation of Ras oncogene PCR product; and the sensor optically detected point mutations at DNA concentrations of 0.2 - 196 nM following a 20 minute hybridization. Lower concentrations of  
25 target oligonucleotides could be detected by hybridizing for longer times. The biosensor's small size (350  $\mu$ m o.d.) and the small volume of the individual array elements (20 pL) enable sub-microliter sample volumes to be analyzed, increasing the value of the sensor.

The present invention is not to be restricted in form nor limited in scope except by the claims appended hereto.



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What we claim is:

1. An optical biosensor for selectively detecting an oligonucleotide specie in a fluid sample, said biosensor comprising:

5 a clad optical fiber strand of determinable configuration and dimensions which presents two strand end faces as discrete optic surfaces for introduction and conveyance of light energy;

an oligonucleotide in-situ hybridization zone comprising one specie of single-stranded oligonucleotide disposed as a deposit upon one of said strand end faces of said optical fiber  
10 strand, said deposit of single stranded oligonucleotide within said hybridization zone serving as a deployed, single-specie, fixed probe suitable for selective in-situ hybridization on-demand with a mobile complementary oligonucleotide target specie,

(a) wherein such complementary oligonucleotide target specie as is hybridized in-situ ultimately bears a joined identifying label comprising at least one light  
15 energy absorbing dye of known spectral characteristics, and

(b) wherein the resulting, specie specific, in-situ hybridized oligonucleotide reaction product formed by a complementary oligonucleotide target specie with said fixed probe at said hybridization zone is optically detectable via the spectral characteristics of said joined identifying label concomitantly disposed at said strand end face  
20 surface.

2. An optical biosensor for selectively detecting at least one oligonucleotide species in a fluid sample, said biosensor comprising:

a bundled array comprising a plurality of individually clad, optical fiber strands  
25 disposed co-axially along their lengths and having two discrete array ends each of which is formed of multiple strand end faces, said bundled array being of determinable configuration and dimensions and said two discrete array ends presenting two optic array surfaces for introduction and conveyance of light energy; and

at least one oligonucleotide in-situ hybridization zone each comprising one specie of  
30 single-stranded oligonucleotide disposed as individual specie deposits in aligned organization

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upon a single strand end face at differing spatial positions on one of said optic array end surfaces of said bundled array, the differing spatial positionings for each deposit of single-stranded oligonucleotide specie in aligned organization within said hybridization zone serving as deployed, single-specie, multiple fixed probes suitable for selective in-situ hybridization on-demand with a mobile complementary oligonucleotide target specie.

(a) wherein such complementary oligonucleotide target specie as is hybridized bears a joined identifying label comprising at least one light energy absorbing dye of known spectral characteristics, and

(b) wherein the resulting, species specific, in-situ hybridized oligonucleotide reaction product formed by a complementary oligonucleotide target specie with said multiple fixed probes at said differing spatial positions within said hybridization zone is optically detectable via the spectral characteristics of said joined identifying label concomitantly disposed at said differing spatial positions.

3. An optical biosensor for selectively detecting a plurality of oligonucleotide species concurrently in a fluid sample containing a mixture of different oligonucleotide species, said biosensor comprising:

a preformed, unitary fiber optic array comprising a plurality of individually clad, optical fiber strands disposed co-axially along their lengths and having two discrete optic array ends each of which is formed of multiple strand end faces, said preformed unitary fiber optic array being of determinable configuration and dimensions and said two discrete optic array ends presenting two discrete optic array surfaces for introduction and conveyance of light energy; and

at least one oligonucleotide in-situ hybridization zone comprising a plurality of single stranded oligonucleotide species disposed as individual specie deposits in aligned organization upon multiple strand end faces at differing spatial positions on one of said discrete optic fiber array surfaces of said unitary fiber optic array, the differing spatial positionings for each deposit of single stranded oligonucleotide specie in aligned organization within said in-situ hybridization zone serving as a collective of deployed, specie specific, fixed probes suitable for selective in-situ hybridization on-demand with its mobile

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complementary oligonucleotide target specie in a fluid mixture containing alternative mobile complementary target species,

(a) wherein such complementary oligonucleotide target species as are hybridized in-situ each ultimately bears a joined identifying label comprising at least one light energy absorbing dye of known spectral characteristics, and

(b) wherein the resulting, species specific, in-situ hybridized oligonucleotide reaction products formed by a complementary oligonucleotide target specie with one collective of multiple fixed probes deployed at said differing spatial positions within said hybridization zone is optically detectable via the spectral characteristics of said joined identifying label concomitantly disposed at each of said differing spatial positions, each of the alternative in-situ hybridized oligonucleotide reaction products formed being concurrently optically detectable via the spectral characteristics of said joined identifying label concomitantly disposed at each of said differing spatial positions within said hybridization zone.

15

4. The optical biosensor as recited in claim 1, 2, or 3 further comprising

means for placing said hybridization zone of said biosensor into reactive contact with a fluid sample;

means for introducing light energy to a discrete optic surface of said biosensor such that said biosensor conveys said introduced light and illuminates at least a portion of said in-situ hybridization zone; and

means for detecting light energy emerging from said illuminated in-situ hybridization zone of said biosensor, said detected emerging light energy serving as an optical determination of at least one complementary oligonucleotide target specie in the fluid sample.

25

5. An optical method for selectively detecting at least one oligonucleotide specie in a fluid sample containing a mixture of oligonucleotide species, said optical method comprising the steps of:

obtaining a biosensor comprised of a clad optical fiber strand of determinable configuration and dimensions which presents two strand end faces as discrete optic surfaces

30

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for introduction and conveyance of light energy;

providing an oligonucleotide in-situ hybridization zone comprising one specie of single-stranded oligonucleotide disposed as a deposit upon one of said strand end faces of said optical fiber strand, said deposit of single stranded oligonucleotide within said hybridization

5 zone serving as a deployed, single-specie, fixed probe suitable for selective in-situ hybridization on-demand with a mobile complementary oligonucleotide target specie,

(a) wherein such complementary oligonucleotide target specie as is hybridized in-situ ultimately bears a joined identifying label comprising at least one light energy absorbing dye of known spectral characteristics, and

10 (b) wherein the resulting, specie specific, in-situ hybridized oligonucleotide reaction product formed by a complementary oligonucleotide target specie with said fixed probe at said hybridization zone is optically detectable via the spectral characteristics of said joined identifying label concomitantly disposed at said strand end face surface.

15 placing said in-situ hybridization zone of said biosensor into reactive contact with a fluid sample containing a mixture of mobile oligonucleotide species;

permitting hybridization to occur in-situ between said fixed probe of said biosensor and such complementary oligonucleotide target species as are present in the fluid sample;

20 joining an identifying label to the complementary oligonucleotide target specie hybridized in-situ;

introducing light energy to a discrete optic array surface of said biosensor such that said individually clad fiber optical strand conveys said introduced light energy and illuminates said fixed probe within said in-situ hybridization zone of said biosensor; and

25 detecting light energy emerging from said illuminated fixed probe within said in-situ hybridization zone of said biosensor, said detected emerging light serving as an optical detection of a specific complementary oligonucleotide target specie in the fluid sample.

6. An optical method for selectively detecting at least one oligonucleotide specie in a fluid sample containing a mixture of oligonucleotide species, said optical method comprising the

30 steps of:

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obtaining a biosensor comprised of a bundle of clad optical fiber strands of determinable configuration and dimensions which presents a bundle of two strand end faces as discrete optic surfaces for introduction and conveyance of light energy;

providing an oligonucleotide in-situ hybridization zone comprising one specie of  
5 single-stranded oligonucleotide disposed as a deposit upon one of said strand end faces of said optical fiber strand bundle array, said deposit of single stranded oligonucleotide within said hybridization zone serving as a deployed, single-specie, fixed probe suitable for selective in-situ hybridization on-demand with a mobile complementary oligonucleotide target specie,

(a) wherein such complementary oligonucleotide target specie as is  
10 hybridized in-situ ultimately bears a joined identifying label comprising at least one light energy absorbing dye of known spectral characteristics, and

(b) wherein the resulting, specie specific, in-situ hybridized oligonucleotide reaction product formed by a complementary oligonucleotide target specie with said fixed probes at said hybridization zones is optically detectable via the spectral  
15 characteristics of said joined identifying label concomitantly disposed at said strand end face surfaces;

placing said fixed probe within said in-situ hybridization zone of said biosensor into reactive contact with a fluid sample containing a mixture of mobile oligonucleotide species each bearing a joined identifying label;

20 permitting hybridization to occur in-situ between said fixed probe of said biosensor and such complementary oligonucleotide target species bearing a joined identifying label as are present in the fluid sample;

introducing light energy to one of said discrete optic array surface of said biosensor such that said individually clad fiber optical strand conveys said introduced light energy and  
25 illuminates said fixed probe within said in-situ hybridization zone of said biosensor; and

detecting light energy emerging from said illuminated fixed probe within said in-situ hybridization zone of said sensor, said detected emerging light serving as an optical detection of a specific complementary oligonucleotide target specie in the fluid sample.

30 7. An optical method for selectively detecting at least one oligonucleotide specie in a fluid

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sample containing a mixture of oligonucleotide species, said optical method comprising the steps of:

obtaining a biosensor comprised of

an integrated optic array comprising a plurality of individually clad, optical  
5 fiber strands disposed co-axially along their lengths and having two discrete optic array ends  
each of which is formed of multiple strand end faces, said integrated optic array being of  
determinable configuration and dimensions and said two discrete array ends presenting two  
optic array surfaces for introduction and conveyance of light energy, and  
at least one oligonucleotide in-situ hybridization zone comprising not less than  
10 one specie of single-stranded oligonucleotide disposed as individual specie deposits in aligned  
organization upon multiple strand end faces at differing spatial positions on one of said  
discrete optic array surfaces of said integrated optic array, the differing spatial positionings for  
each deposit of single stranded oligonucleotide specie in aligned organization within said  
hybridization zone serving as deployed, single specie, multiple fixed probes suitable for  
15 selective in-situ hybridization on-demand with a mobile complementary oligonucleotide target  
specie,

(a) wherein such complementary oligonucleotide target specie as is hybridized in-situ  
ultimately bears a joined identifying label comprising at least one light energy absorbing dye  
of known spectral characteristics, and

20 (b) wherein the resulting, species specific, in-situ hybridized oligonucleotide reaction  
product formed by a complementary oligonucleotide target specie with said fixed probes  
deployed at said differing spatial positions within said hybridization zone is optically  
detectable via the spectral characteristics of said joined identifying label concomitantly  
disposed at said differing spatial positions;

25 placing said in-situ hybridization zone of said biosensor into reactive contact with a  
fluid sample containing a mixture of mobile oligonucleotide species;

permitting hybridization to occur in-situ between said multiple fixed probes of said  
biosensor and such complementary oligonucleotide target species as are present in the fluid  
sample;

30 joining an identifying label to the complementary oligonucleotide target specie

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hybridized in-situ;

introducing light energy to a discrete optic array surface of said biosensor such that a plurality of individually clad fiber optical strands convey said introduced light energy concurrently and illuminate at least a portion said multiple fixed probes at differing spatial positions within said in-situ hybridization zone of said biosensor; and

detecting light energy emerging from said illuminated multiple fixed probes at differing spatial positions within said in-situ hybridization zone of said biosensor, said detected emerging light energy serving as an optical detection of at least one specific complementary oligonucleotide target specie in the fluid sample.

10

8. An optical method for selectively detecting at least one oligonucleotide specie in a fluid sample containing a mixture of oligonucleotide species, said optical method comprising steps of:

obtaining a biosensor comprised of

15

an integrated optic array comprising a plurality of individually clad, optical fiber strands disposed co-axially along their lengths and having two discrete optic array ends each of which is formed of multiple strand end faces, said integrated optic array being of determinable configuration and dimensions and said two discrete array ends presenting two optic array surfaces for introduction and conveyance of light energy, and

20

at least one oligonucleotide in-situ hybridization zone comprising one single stranded oligonucleotide species disposed as individual specie deposits in aligned organization at differing spatial positions on one of said discrete optic fiber array surfaces of said integrated optic array, the differing spatial positionings for each deposit of single stranded oligonucleotide specie in aligned organization within said in-situ hybridization zone

25

serving as deployed, single strand, multiple fixed probes suitable for selective in-situ hybridization on-demand with a mobile complementary oligonucleotide target specie,

(a) wherein such complementary oligonucleotide target specie as is hybridized in-situ ultimately bears a joined identifying label comprising at least one light energy absorbing dye of known spectral characteristics, and

30

(b) wherein the resulting, species selective, in-situ hybridized oligonucleotide reaction

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product formed by a complementary oligonucleotide target specie with said fixed probes deployed at said differing spatial positions within the hybridization zone of said biosensor is optically detectable via the spectral characteristics of said joined identifying label concomitantly disposed at said differing spatial positions;

5        placing said multiple fixed probes within said in-situ hybridization zone of said biosensor into reactive contact with a fluid sample containing a mixture of mobile oligonucleotide species each bearing a joined identifying label;

          permitting hybridization to occur in-situ between said multiple fixed probes of said biosensor and such complementary oligonucleotide target species bearing a joined identifying  
10    label as are present in the fluid sample;

          introducing light energy to one of said discrete optic array surfaces of said biosensor such that a plurality of individually clad fiber optical strands convey said introduced light energy concurrently and illuminate at least a portion of said multiple fixed probes at differing spatial positions within said in-situ hybridization zone of said biosensor; and

15        detecting light energy emerging from said illuminated multiple fixed probes at differing spatial positions within said in-situ hybridization zone of said sensor, said detected emerging light energy serving as an optical detection of at least one complementary oligonucleotide target specie in the fluid sample.



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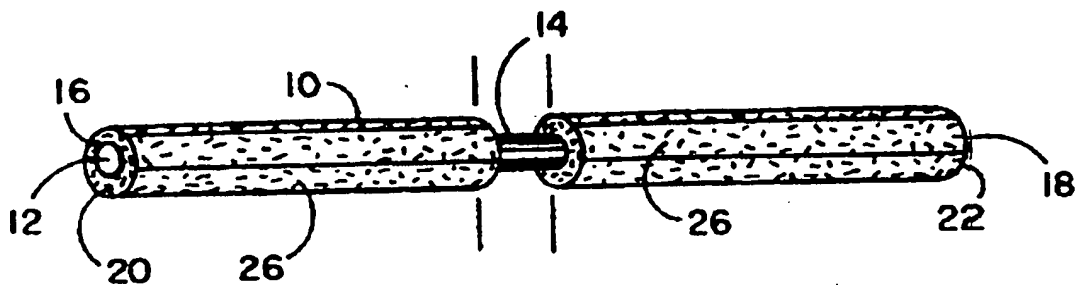


FIG. 1

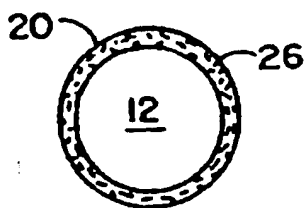


FIG. 2A

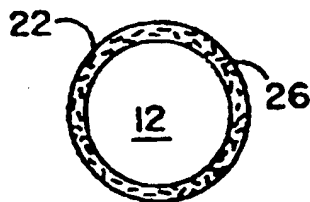


FIG. 2B

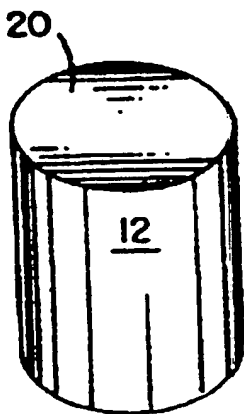


FIG. 3A

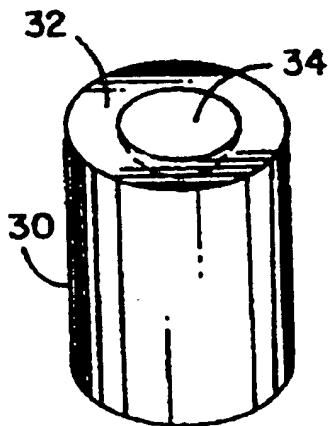


FIG. 3B

FIG. 4

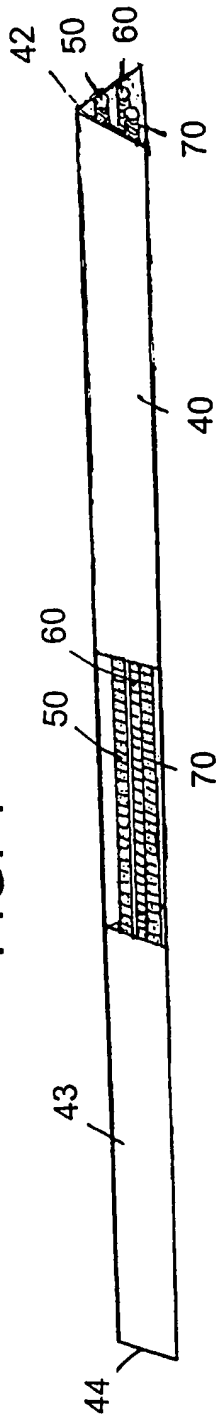


FIG. 5B

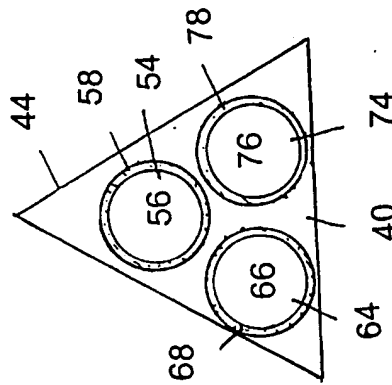
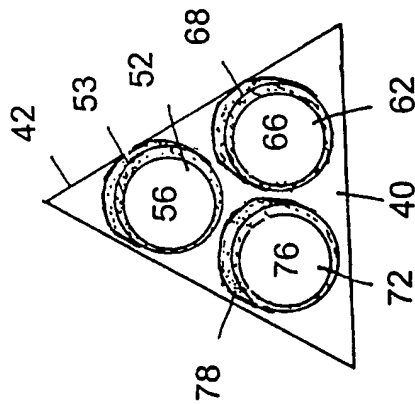


FIG. 5A



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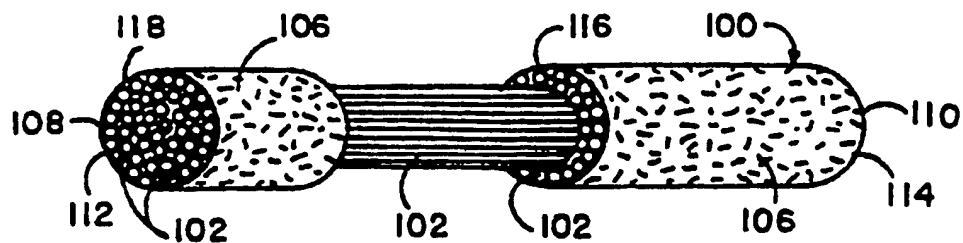


FIG. 6

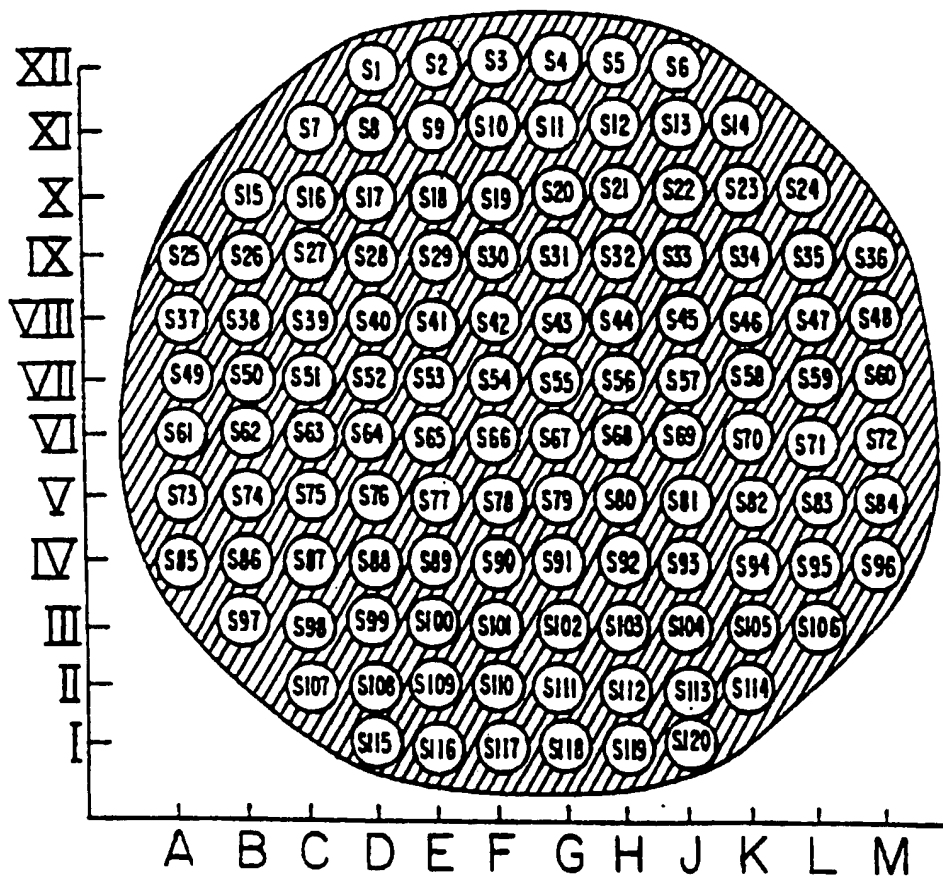


FIG. 7

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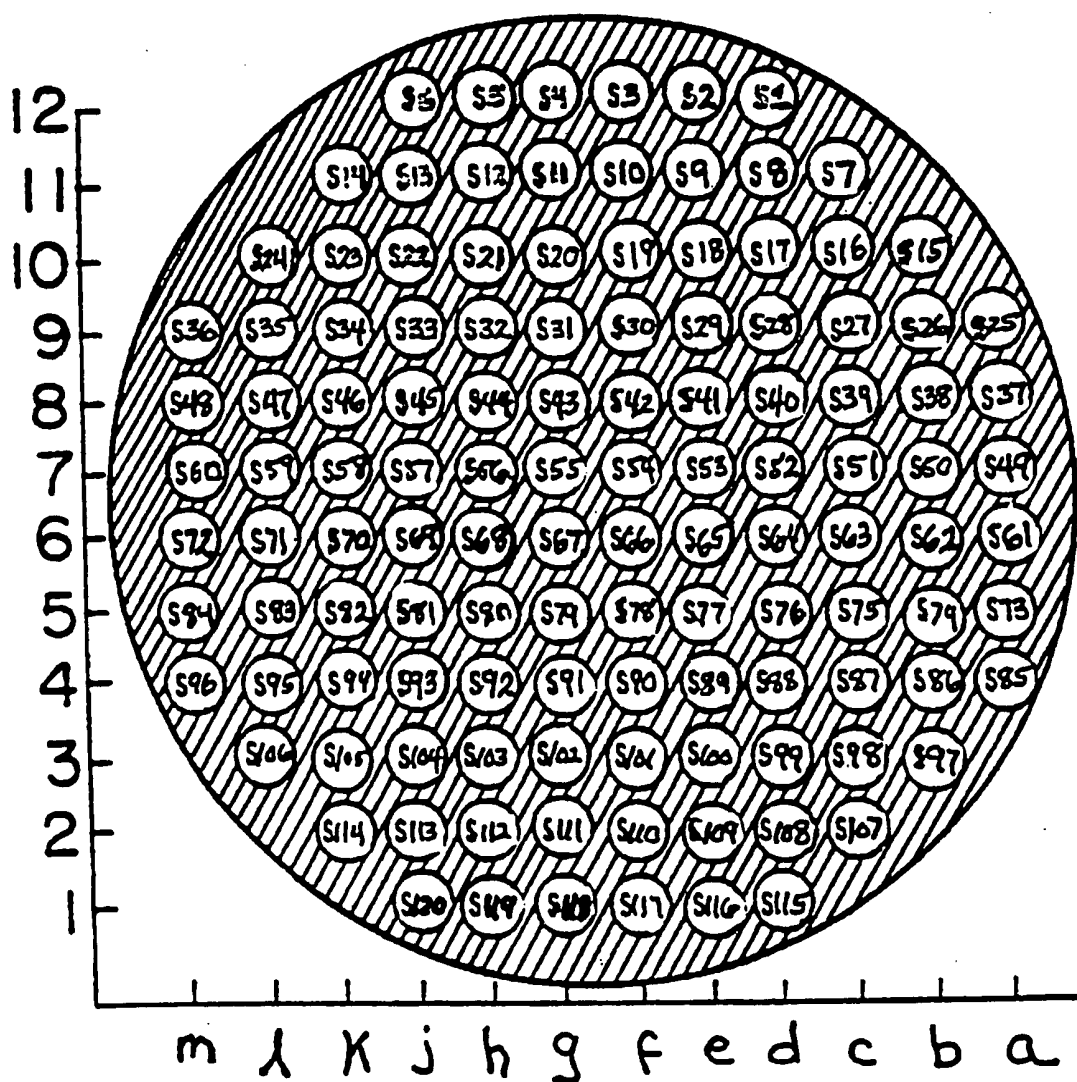


FIG. 8

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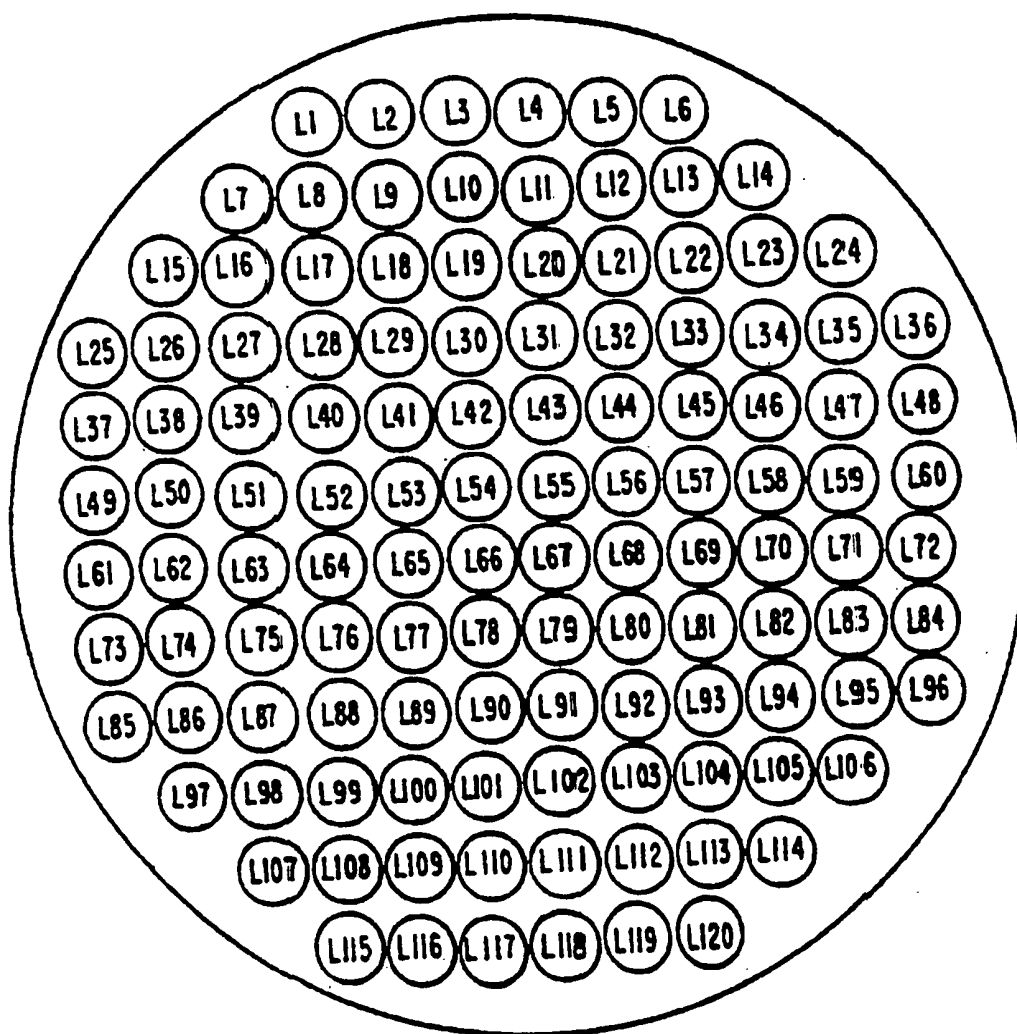


FIG. 9

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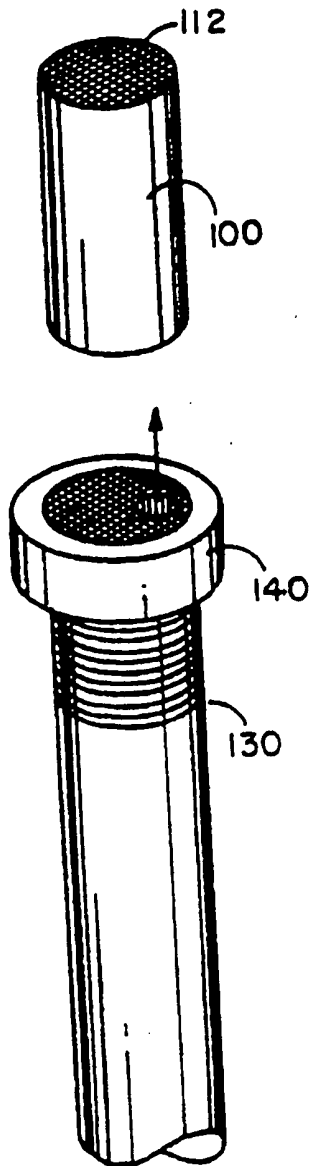


FIG. 10

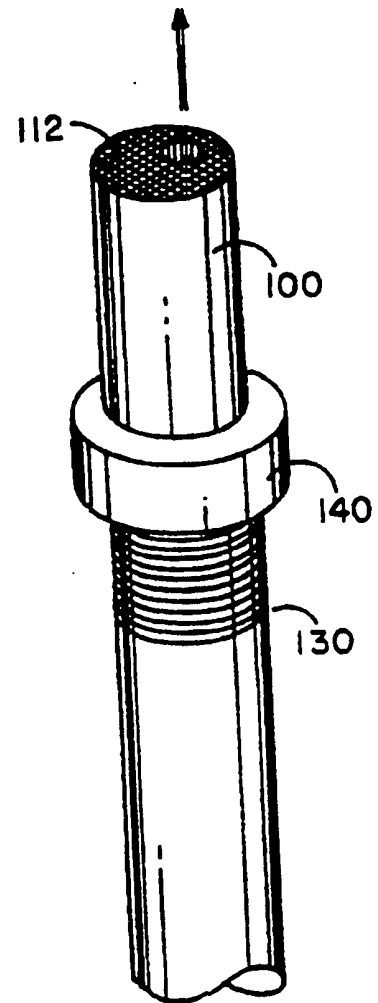


FIG. 11

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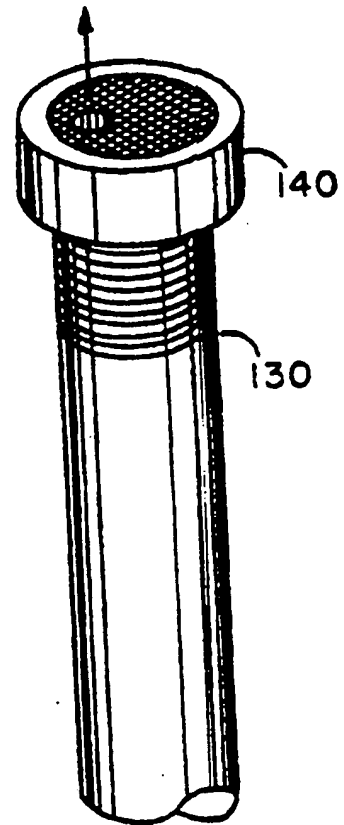
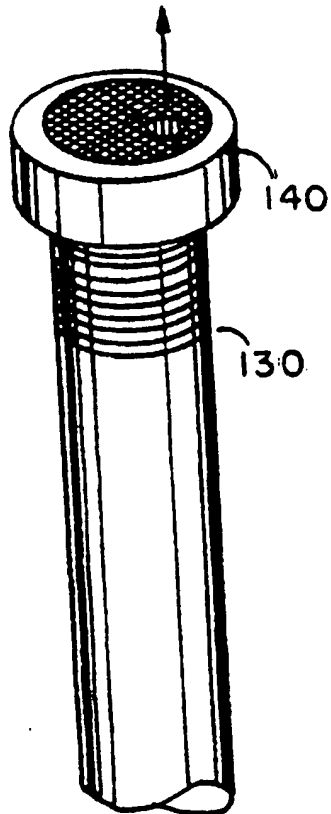
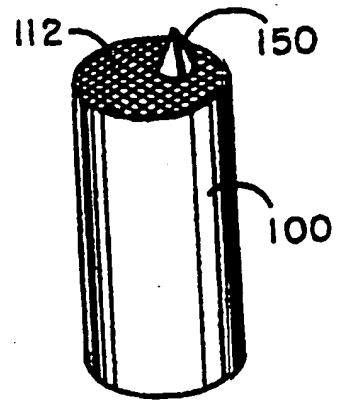
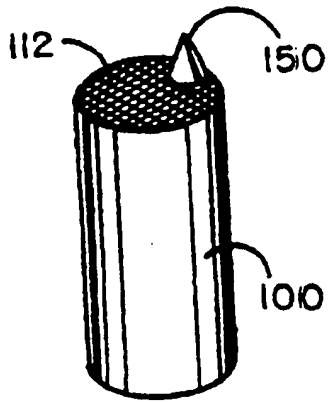


FIG. 12

FIG. 13

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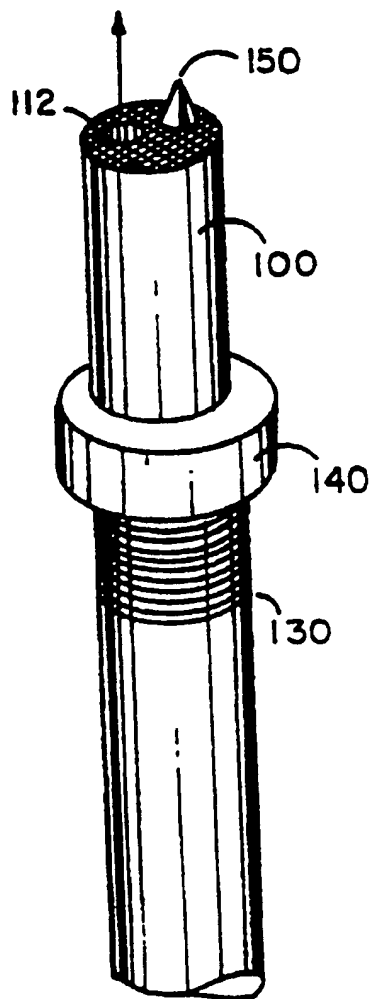


FIG. 14

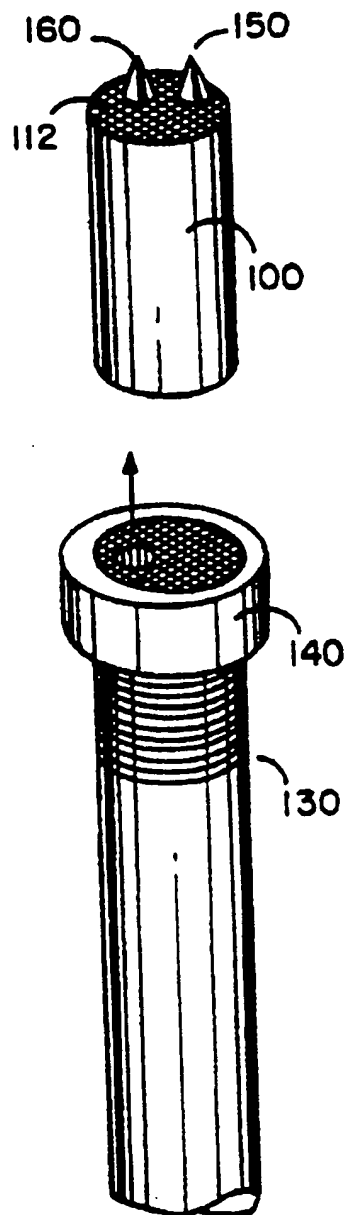


FIG. 15



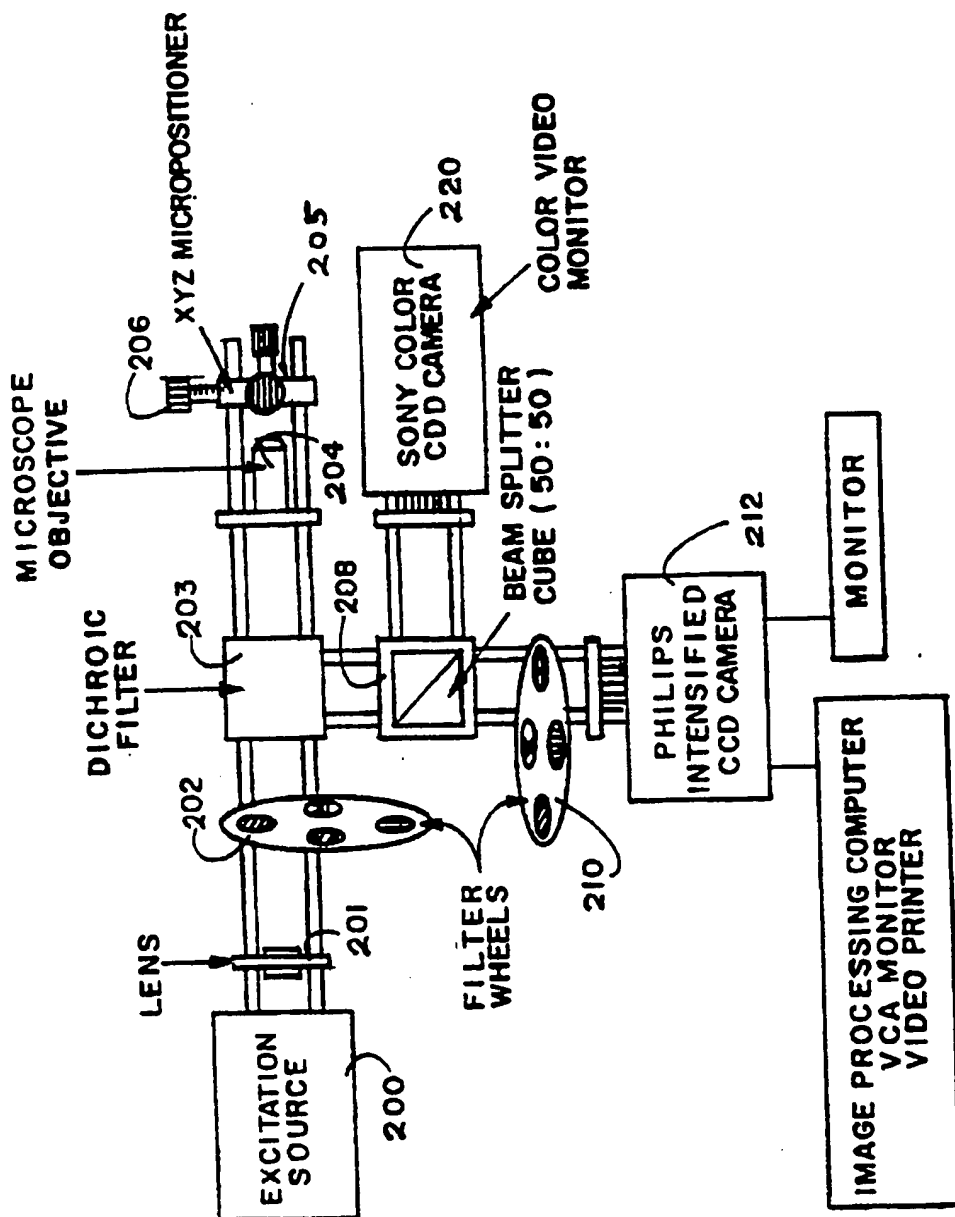


FIG. 16

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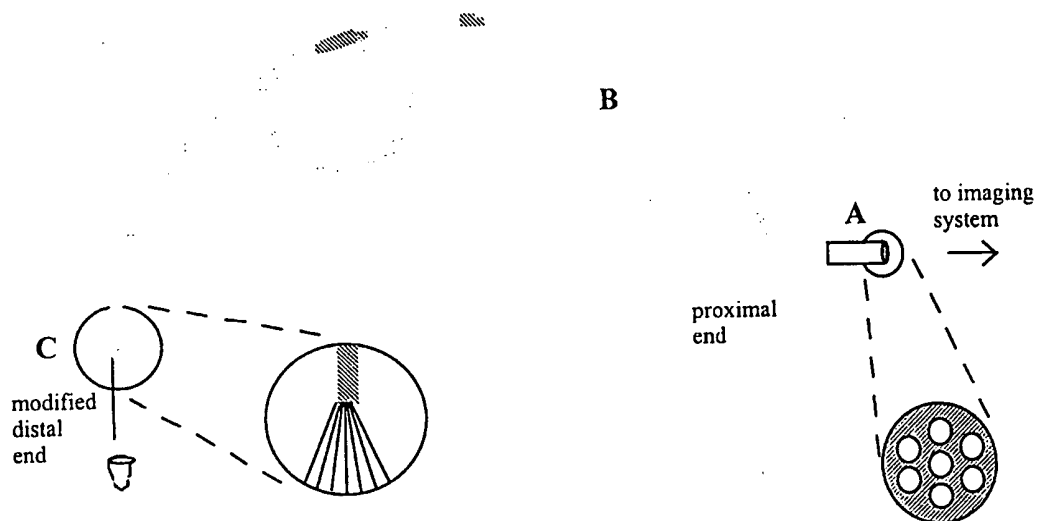


FIG. 17

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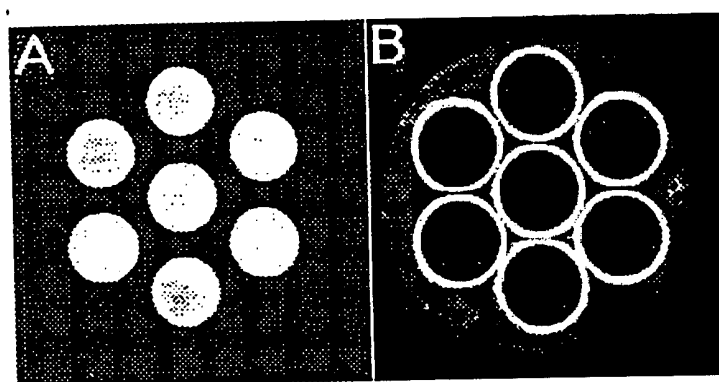


FIG. 18

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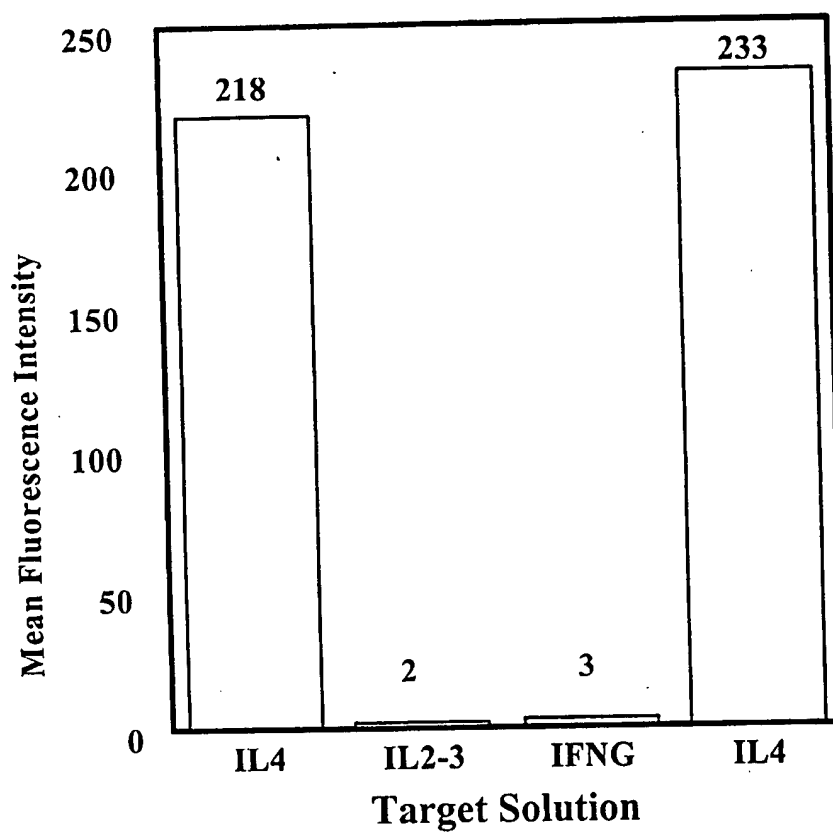


FIG. 19

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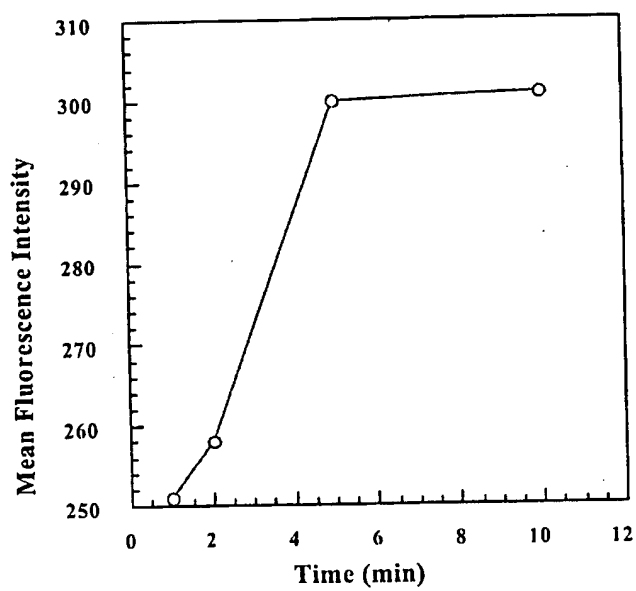


FIG. 20

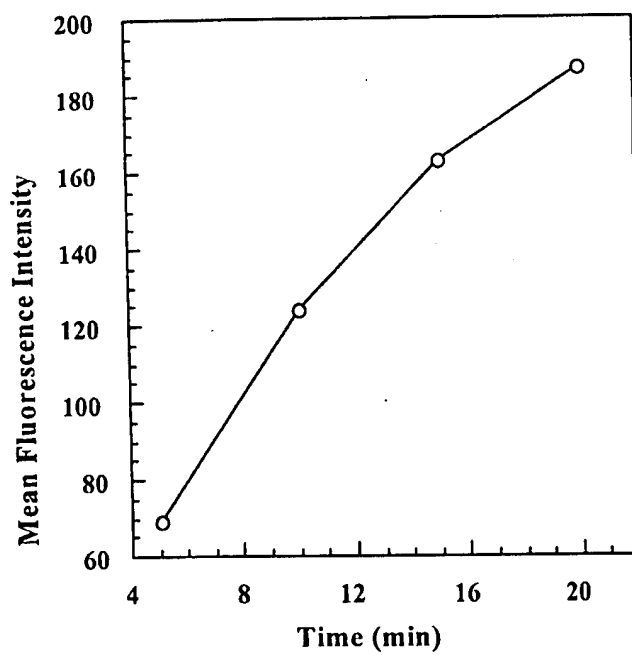


FIG. 21

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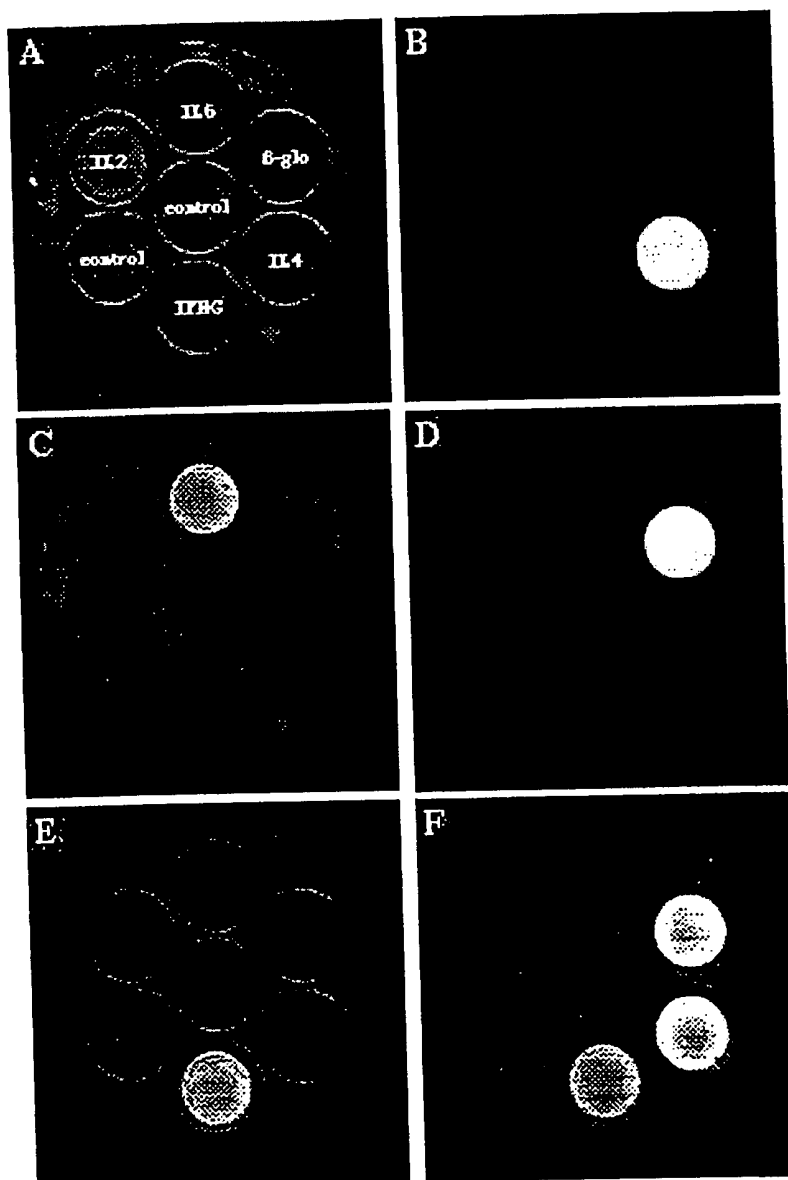


FIG. 22

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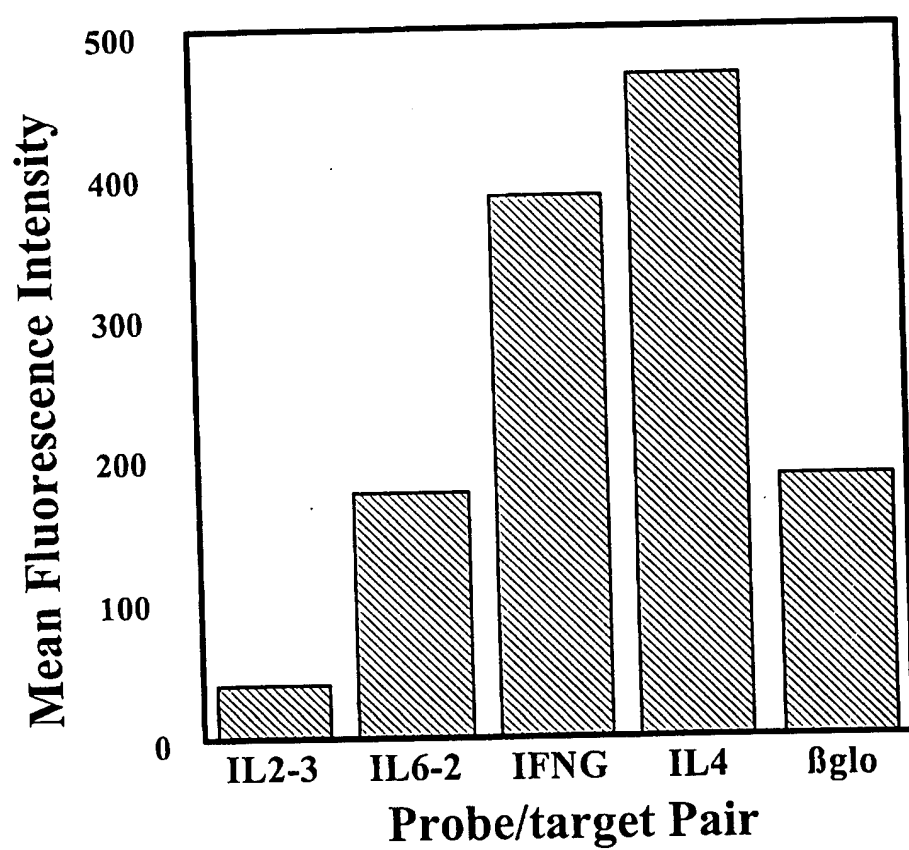


FIG. 23

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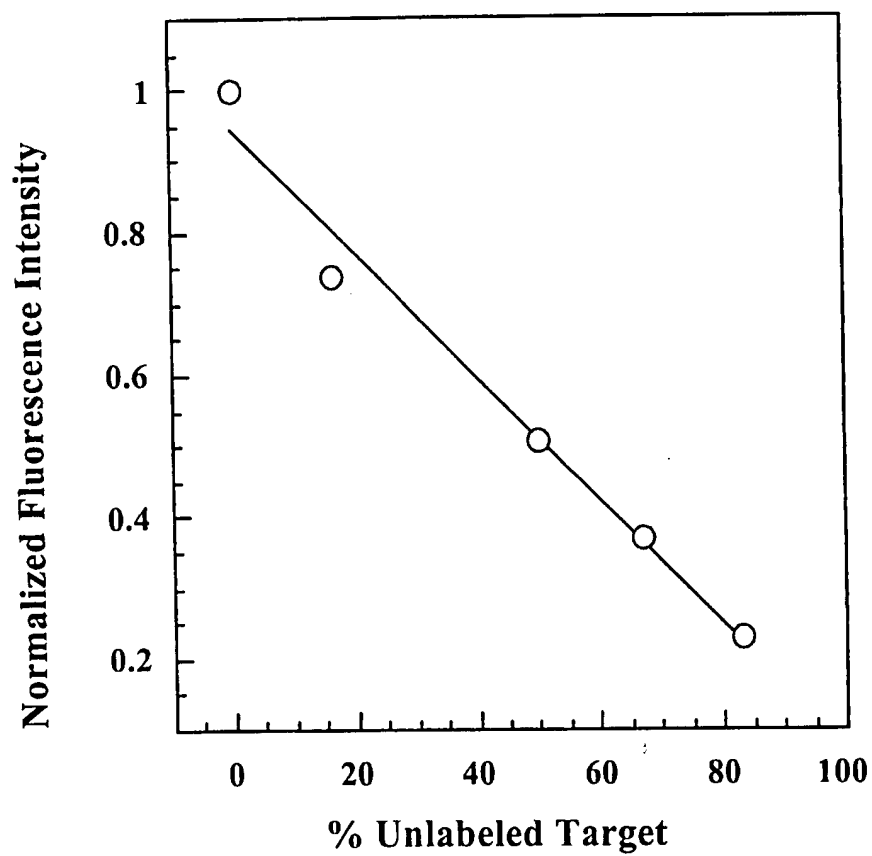


FIG. 24



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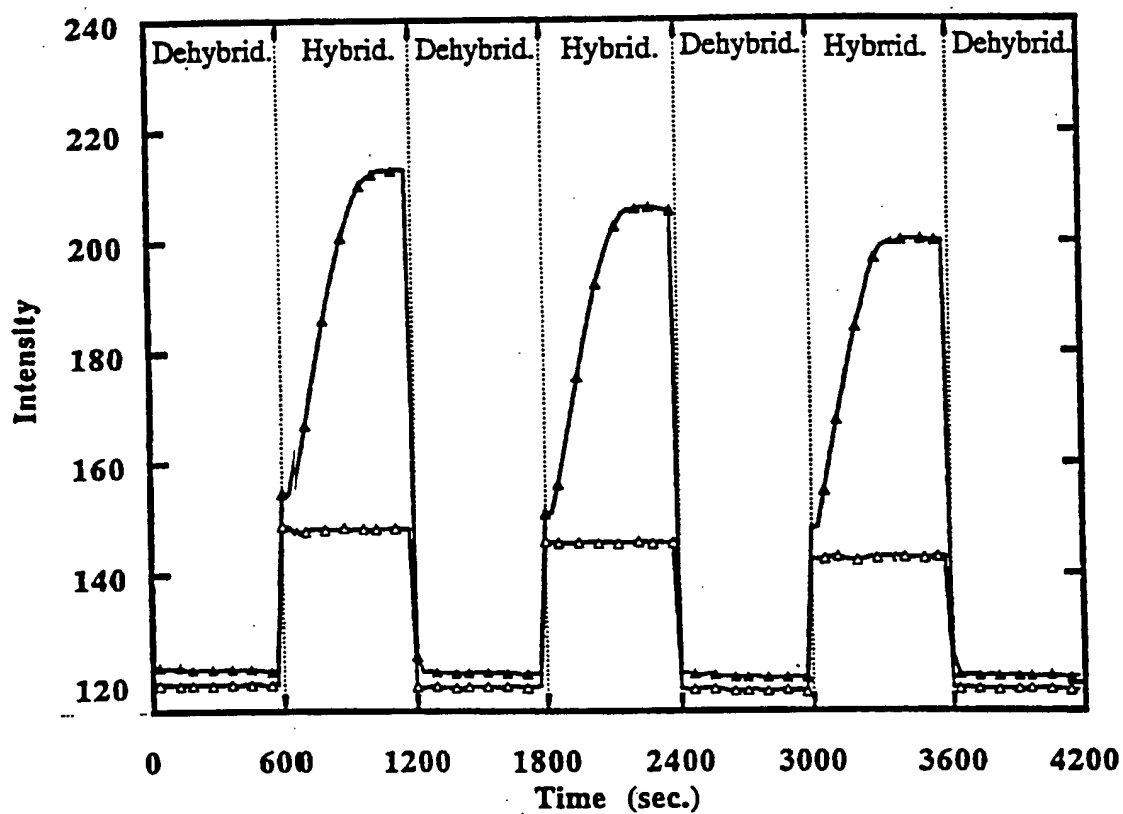


FIG. 25

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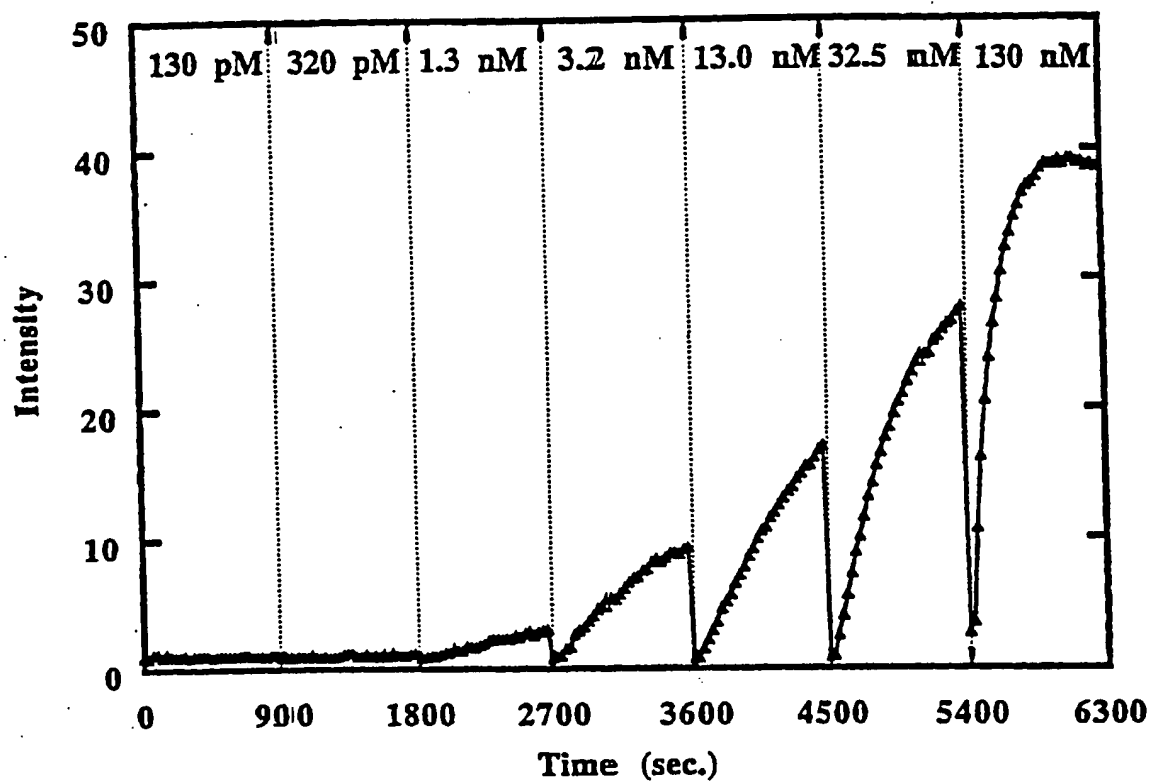


FIG. 26

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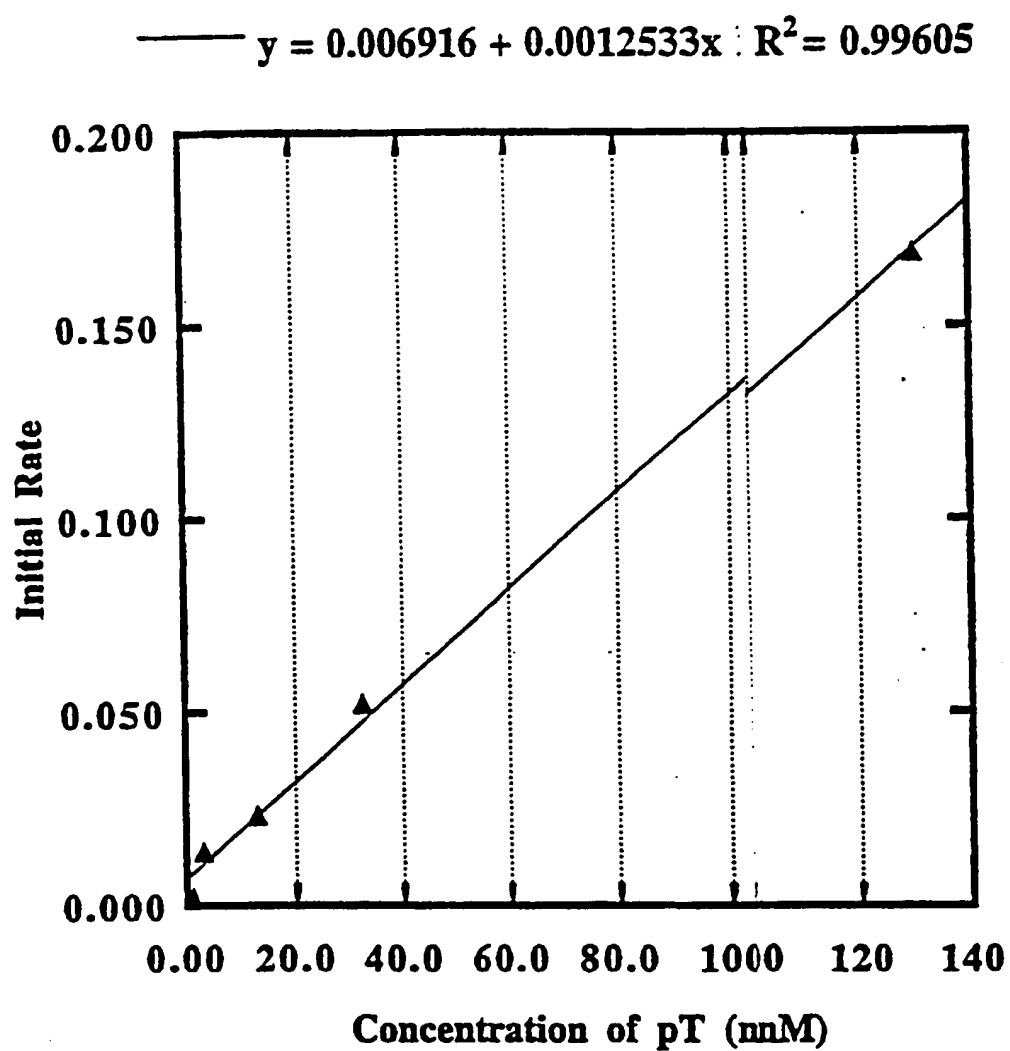


FIG. 27

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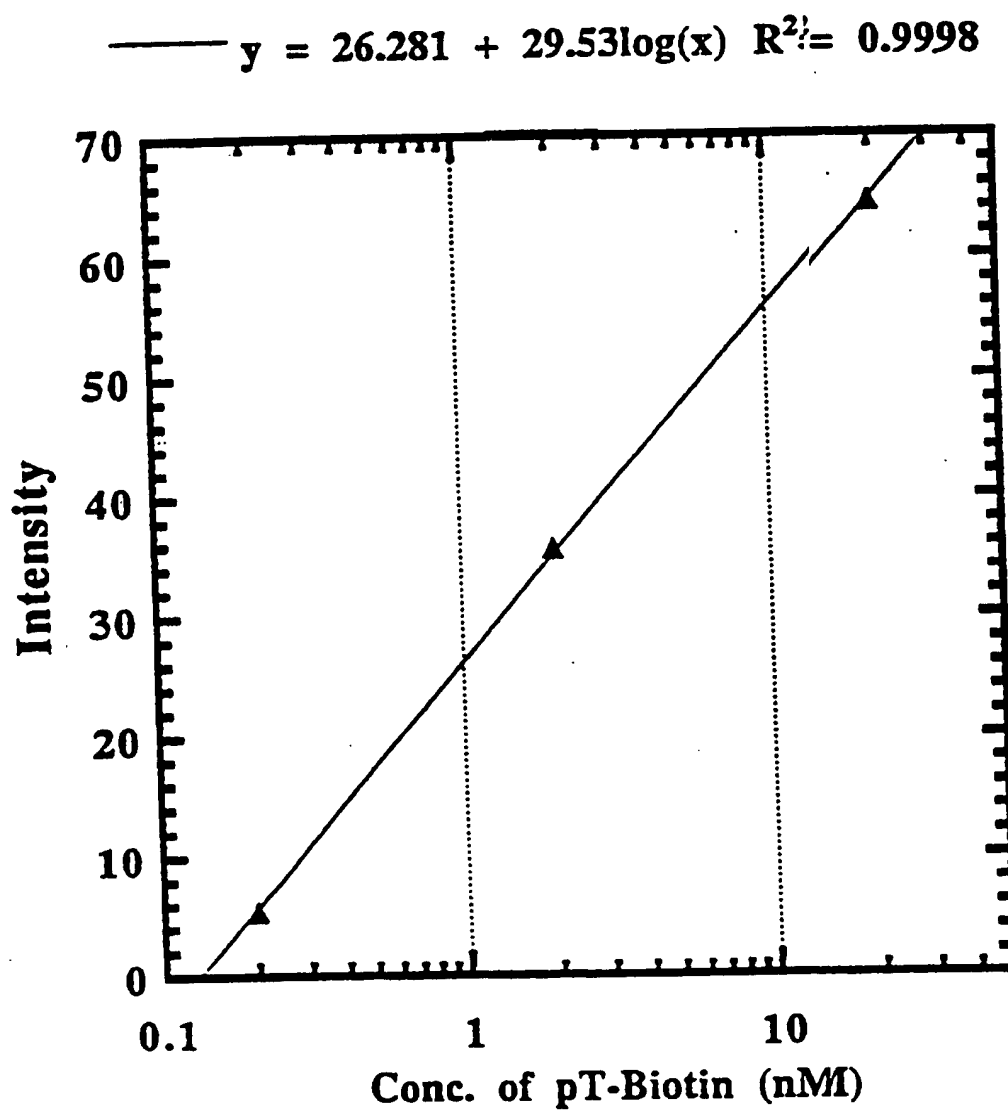


FIG. 28

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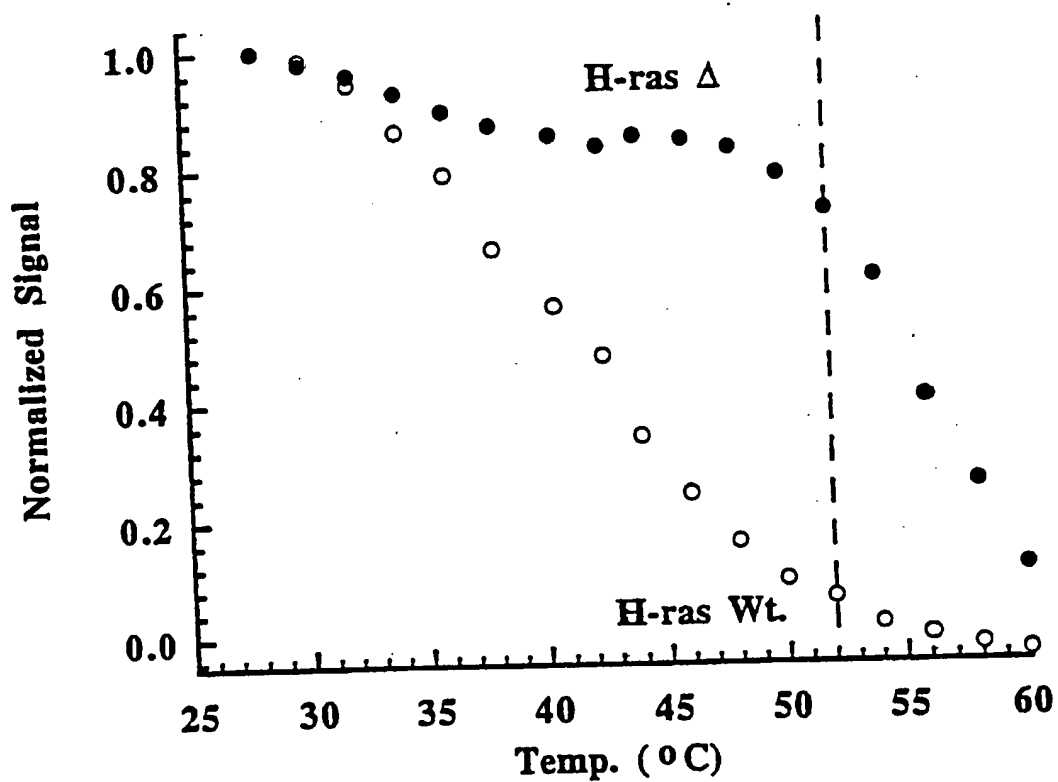


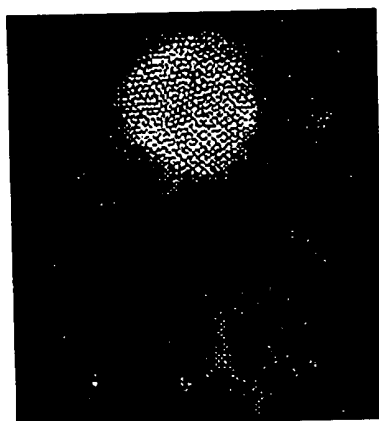
FIG. 29

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28° C

FIG. 30A



54° C

FIG. 30B

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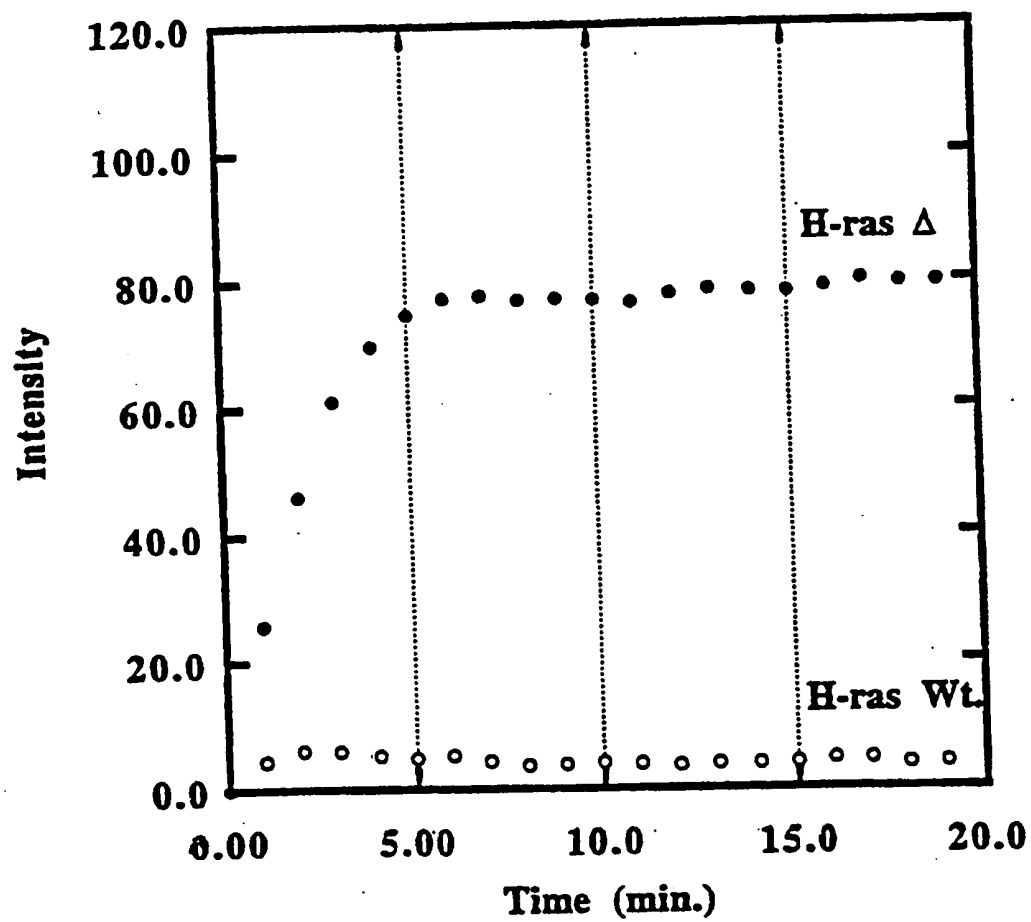


FIG. 31

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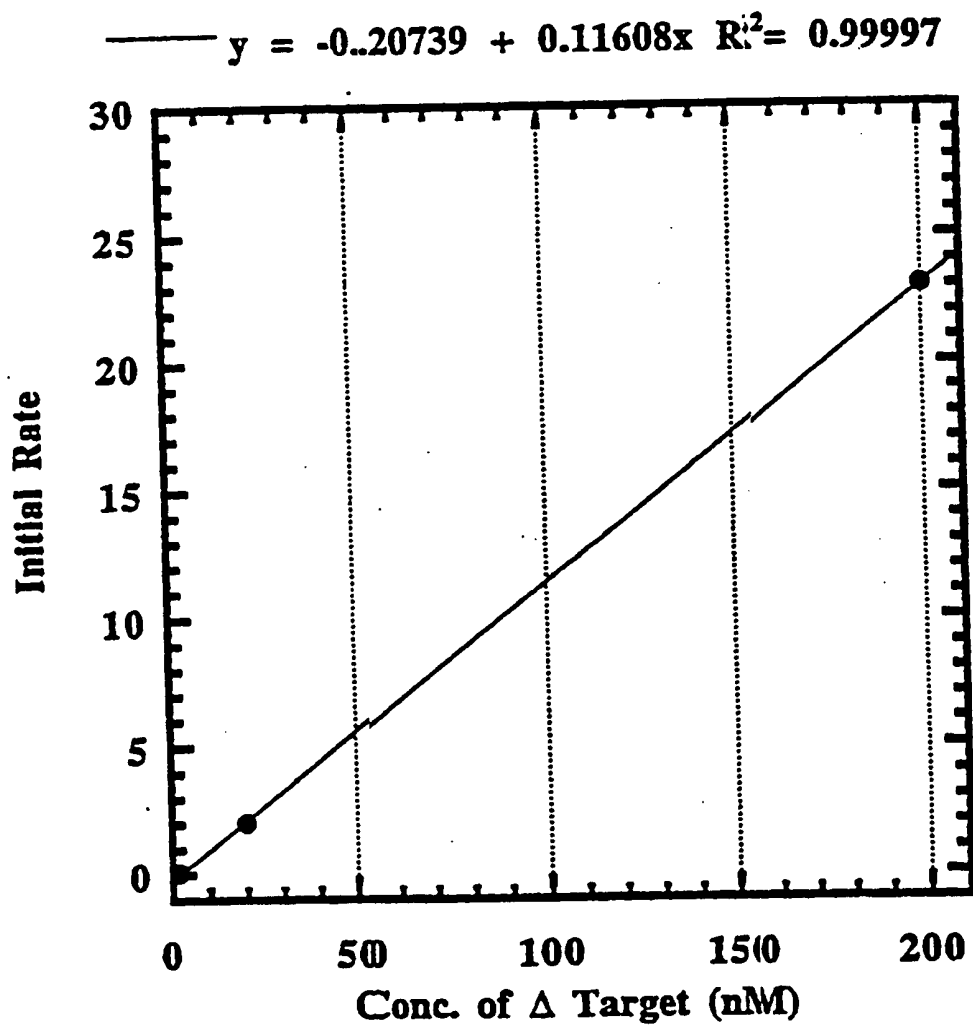


FIG. 32



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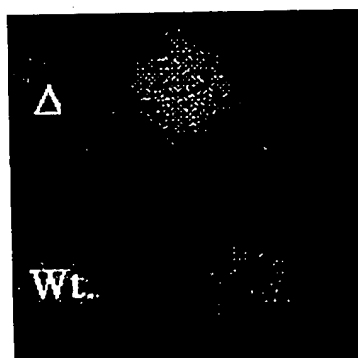


FIG. 33

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<b>(5) International Patent Classification <sup>7</sup>:</b> <b>G01N 33/543</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 00/48000</b> <b>(43) International Publication Date:</b> 17 August 2000 (17.08.00)
<b>(21) International Application Number:</b> PCT/US00/03370 <b>(22) International Filing Date:</b> 9 February 2000 (09.02.00)  <b>(30) Priority Data:</b> 60/119,343 9 February 1999 (09.02.99) US  <b>(71) Applicant:</b> ILLUMINA, INC. [US/US]; Suite 200, 9390 Towne Centre Drive, San Diego, CA 92121 (US).  <b>(72) Inventors:</b> CZARNIK, Anthony, W.; 11017 Camino Abrojo, San Diego, CA 92127 (US). WALT, David, R.; 4 Candlewick Close, Lexington, MA 02173 (US). STUELPNAGEL, John, R.; 38 Briggs Avenue, Encinitas, CA 92024 (US).  <b>(74) Agents:</b> BREZNER, David, J. et al.; Flehr, Hohbach, Test Albritton & Herbert LLP, 4 Embarcadero Center, Suite 3400, San Francisco, CA 94111-4187 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> INTRABEAD SCREENING METHODS AND COMPOSITIONS		
<b>(57) Abstract</b>  The invention relates to the use of porous beads to screen candidate bioactive agents for the ability to interact or effect a target substance. The screen is performed in the interior and exterior surface of the bead, allowing extremely small sample sizes and concentration of reagents, while retarding evaporation.		

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## INTRABEAD SCREENING METHODS AND COMPOSITIONS

## FIELD OF THE INVENTION

5 The invention relates to the use of porous beads to screen candidate bioactive agents for the ability to interact or effect a target substance. The screen is performed in the interior and exterior surface of the bead, allowing extremely small sample sizes and concentration of reagents, while retarding evaporation.

## BACKGROUND OF THE INVENTION

10 Traditional approaches to identify and characterize new and useful drug candidates include the isolation of natural products or synthetic preparation, followed by testing against either known or unknown targets. See for example WO 94/24314, Gallop et al., J. Med. Chem. 37(9):1233 (1994); Gallop et al., J. Med. Chem. 37(10):1385 (1994); Ellman, Acc. Chem. Res. 29:132 (1996); Gordon et al., E. J. Med. Chem. 30:388s (1994); Gordon et al., Acc. Chem. Res. 29:144 (1996); WO 95/12608, all of which are incorporated by reference.

15 The screening of these libraries is done in a variety of ways. One approach involves attachment to beads and visualization with dyes; see Neslter et al., Bioorg. Med. Chem. Lett. 6(12):1327 (1996). Another approach has utilized beads and fluorescence activated cell sorting (FACS); see Needles et al., PNAS USA 90:10700 (1993), and Vetter et al., Bioconjugate Chem. 6:319 (1995).

20 In addition, there are a number of assays and sensors for the detection of the presence and/or concentration of specific substances in fluids and gases. Many of these rely on specific ligand/antiligand reactions as the mechanism of detection. That is, pairs of substances (i.e. the binding pairs or ligand/antiligands) are known to bind to each other, while binding little or not at all to other substances. This has been the focus of a number of techniques that utilize these binding pairs for the detection of the complexes. These generally are done by labeling one component of the complex in some way, so as to make the entire complex detectable, using, for example, radioisotopes,  
25 fluorescent and other optically active molecules, enzymes, etc.

Of particular use in these sensors are detection mechanisms utilizing luminescence. Recently, the use of optical fibers and optical fiber strands in combination with light absorbing dyes for chemical analytical determinations has undergone rapid development, particularly within the last decade. The use of optical fibers for such purposes and techniques is described by Milanovich et al., "Novel Optical Fiber Techniques For Medical Application", Proceedings of the SPIE 28th Annual International Technical Symposium On Optics and Electro-Optics, Volume 494, 1980; Seitz, W.R., "Chemical Sensors Based On Immobilized Indicators and Fiber Optics" in *C.R.C. Critical Reviews In Analytical Chemistry*, Vol. 19, 1988, pp. 135-173; Wolfbeis, O.S., "Fiber Optical Fluorosensors In Analytical Chemistry" in *Molecular Luminescence Spectroscopy, Methods and Applications* (S. G. Schulman, editor), Wiley & Sons, New York (1988); Angel, S.M., *Spectroscopy* 2 (4):38 (1987); Walt, et al., "Chemical Sensors and Microinstrumentation", *ACS Symposium Series*, Vol. 403, 1989, p. 252, and Wolfbeis, O.S., *Fiber Optic Chemical Sensors*, Ed. CRC Press, Boca Raton, FL, 1991, 2nd Volume.

When using an optical fiber in an *in vitro/in vivo* sensor, one or more light absorbing dyes are located near its distal end. Typically, light from an appropriate source is used to illuminate the dyes through the fiber's proximal end. The light propagates along the length of the optical fiber; and a portion of this propagated light exits the distal end and is absorbed by the dyes. The light absorbing dye may or may not be immobilized; may or may not be directly attached to the optical fiber itself; may or may not be suspended in a fluid sample containing one or more analytes of interest; and may or may not be retainable for subsequent use in a second optical determination.

Once the light has been absorbed by the dye, some light of varying wavelength and intensity returns, conveyed through either the same fiber or collection fiber(s) to a detection system where it is observed and measured. The interactions between the light conveyed by the optical fiber and the properties of the light absorbing dye provide an optical basis for both qualitative and quantitative determinations.

Of the many different classes of light absorbing dyes which conventionally are employed with bundles of fiber strands and optical fibers for different analytical purposes are those more common compositions that emit light after absorption termed "fluorophores" and those which absorb light and internally convert the absorbed light to heat, rather than emit it as light, termed "chromophores."

Fluorescence is a physical phenomenon based upon the ability of some molecules to absorb light (photons) at specified wavelengths and then emit light of a longer wavelength and at a lower energy. Substances able to fluoresce share a number of common characteristics: the ability to absorb light energy at one wavelength  $\lambda_{ex}$ ; reach an excited energy state; and subsequently emit light at another light wavelength,  $\lambda_{em}$ . The absorption and fluorescence emission spectra are individual for each fluorophore and are often graphically represented as two separate curves that are slightly overlapping. The same fluorescence emission spectrum is generally observed irrespective of the wavelength of the exciting light and, accordingly, the wavelength and energy of the exciting light may be varied within

limits; but the light emitted by the fluorophore will always provide the same emission spectrum under a given set of conditions. Finally, the strength of the fluorescence signal may be measured as the quantum yield of light emitted. The fluorescence quantum yield is the ratio of the number of photons emitted in comparison to the number of photons initially absorbed by the fluorophore. For more detailed information regarding each of these characteristics, the following references are recommended: Lakowicz, J. R., *Principles of Fluorescence Spectroscopy*, Plenum Press, New York, 1983; Freifelder, D., *Physical Biochemistry*, second edition, W. H. Freeman and Company, New York, 1982; "Molecular Luminescence Spectroscopy Methods and Applications: Part I" (S.G. Schulman, editor) in *Chemical Analysis*, vol. 77, Wiley & Sons, Inc., 1985; *The Theory of Luminescence*, Stepanov and Gribkovskii, Iliffe Books, Ltd., London, 1968.

In comparison, substances which absorb light and do not fluoresce usually convert the light into heat or kinetic energy. The ability to internally convert the absorbed light identifies the dye as a "chromophore." Dyes which absorb light energy as chromophores do so at individual wavelengths of energy and are characterized by a distinctive molar absorption coefficient at that wavelength. Chemical analysis employing fiber optic strands and absorption spectroscopy using visible and ultraviolet light wavelengths in combination with the absorption coefficient allow for the determination of concentration for specific analyses of interest by spectral measurement. The most common use of absorbance measurement via optical fibers is to determine concentration which is calculated in accordance with Beers' law; accordingly, at a single absorbance wavelength, the greater the quantity of the composition which absorbs light energy at a given wavelength, the greater the optical density for the sample. In this way, the total quantity of light absorbed directly correlates with the quantity of the composition in the sample.

Many of the recent improvements employing optical fiber sensors in both qualitative and quantitative analytical determinations concern the desirability of depositing and/or immobilizing various light absorbing dyes at the distal end of the optical fiber. In this manner, a variety of different optical fiber chemical sensors and methods have been reported for specific analytical determinations and applications such as pH measurement, oxygen detection, and carbon dioxide analyses. These developments are exemplified by the following publications: Freeman, et al., *Anal. Chem.* 53:98 (1983); Lippitsch et al., *Anal. Chem. Acta.* 205:1, (1988); Wolfbeis et al., *Anal. Chem.* 60:2028 (1988); Jordan, et al., *Anal. Chem.* 59:437 (1987); Lubbeis et al., *Sens. Actuators* 1983; Munkholm et al., *Talanta* 35:109 (1988); Munkholm et al., *Anal. Chem.* 58:1427 (1986); Seitz, W. R., *Anal. Chem.* 56:16A-34A (1984); Peterson, et al., *Anal. Chem.* 52:864 (1980); Saari, et al., *Anal. Chem.* 54:821 (1982); Saari, et al., *Anal. Chem.* 55:667 (1983); Zhujun et al., *Anal. Chem. Acta.* 160:47 (1984); Schwab, et al., *Anal. Chem.* 56:2199 (1984); Wolfbeis, O.S., "Fiber Optic Chemical Sensors", Ed. CRC Press, Boca Raton, FL, 1991, 2nd Volume; and Pantano, P., Walt, D.R., *Anal. Chem.*, 481A-487A, Vol. 67, (1995).

More recently, fiber optic sensors have been constructed that permit the use of multiple dyes with a single, discrete fiber optic bundle. U.S. Pat. Nos. 5,244,636 and 5,250,264 to Walt, *et al.* disclose systems for affixing multiple, different dyes on the distal end of the bundle, the teachings of each of these patents being incorporated herein by this reference. The disclosed configurations enable  
5 separate optical fibers of the bundle to optically access individual dyes. This avoids the problem of deconvolving the separate signals in the returning light from each dye, which arises when the signals from two or more dyes are combined, each dye being sensitive to a different analyte, and there is significant overlap in the dyes' emission spectra.

U.S.S.N.s 08/818,199 and 09/151,877 describe array compositions that utilize microspheres or beads  
10 on a surface of a substrate, for example on a terminal end of a fiber optic bundle, with each individual fiber comprising a bead containing an optical signature. Since the beads go down randomly, a unique optical signature is needed to "decode" the array; i.e. after the array is made, a correlation of the location of an individual site on the array with the bead or bioactive agent at that particular site can be made. This means that the beads may be randomly distributed on the array, a fast and inexpensive  
15 process as compared to either the *in situ* synthesis or spotting techniques of the prior art. Once the array is loaded with the beads, the array can be decoded, or can be used, with full or partial decoding occurring after testing, as is described in U.S.S.N.s 60/090,473 and 09/189,543.

Accordingly, it is an object of the present invention to provide compositions and methods for the screening of candidate bioactive agents for their ability to interact or effect a target analyte.

## 20 SUMMARY OF THE INVENTION

In accordance with the objects outlined above, the present invention provides array compositions comprising a substrate with a surface comprising discrete sites, and a population of microspheres comprising at least a first and a second subpopulation. Each subpopulation comprises a candidate  
25 bioactive agent linked to the microspheres via a scissile linkage, wherein the microspheres are distributed on the surface. The microspheres may optionally further comprise an identifier moiety.

In an additional aspect, the invention provides array compositions wherein the amount of candidate agent on the first subpopulation is different from the amount of candidate agent on the second subpopulation, such that different concentrations of candidate agents can be assayed.

In a further aspect, the invention provides assay methods for detecting the binding of a candidate  
30 bioactive agent to a target analyte comprising adding a solution comprising at least one target analyte to a plurality of microspheres comprising at least a first and a second subpopulation, wherein each subpopulation comprises a candidate bioactive agent linked to the microspheres via a scissile linkage.

The scissile linkage is then cleaved, and the binding of at least one candidate bioactive agent to the target analyte is detected.

#### DETAILED DESCRIPTION OF THE INVENTION

5 The present invention is directed to "in-bead" or "intrabead" screening, wherein assays, particularly binding assays, are run within and on a porous bead. That is, candidate bioactive agents are synthesized on a porous bead, resulting in the candidate agents being attached, frequently via a scissile linkage, to the internal and external surface of a bead. As is more fully described below, these beads can be randomly distributed at discrete sites on a surface, for example a terminus of a fiber optic bundle, such that extremely large numbers of assays can be simultaneously run. The assay  
10 reagents can then be added, such as buffers, co-factors, metal ions and other assay reagents, and the target analyte for which binding is being evaluated, and the candidate bioactive agent is optionally cleaved from the bead, allowing free diffusion through the bead and interaction with the target. Detection of binding is then accomplished in a variety of ways, as is more fully outlined below.

15 Thus the present invention has a number of important advantages, including the use of very low amounts of reagents, a distinct spatial localization of the assays, decreased propensity to diffusion and decreased rates of solvent evaporation. In addition, as is more fully outlined below, by varying the amount of candidate bioactive agent attached to each bead, the concentration of the candidate bioactive agent can be varied, allowing the simultaneous determination of concentration effects. This is quite significant, as it allows the determination of kinetic parameters such as  $K_m$  and  $K_i$  and also  
20 avoids subsequent scale-up, including resynthesis of the agents and reassays.

Accordingly, the present invention provides array compositions. By "array" herein is meant a plurality of candidate agents in an array format; the size of the array will depend on the composition and end use of the array. Arrays containing from about 2 different bioactive agents (i.e. different beads) to many millions can be made, with very large fiber optic arrays being possible. Generally, the array will  
25 comprise from two to as many as a billion or more, depending on the size of the beads and the substrate, as well as the end use of the array, thus very high density, high density, moderate density, low density and very low density arrays may be made. Preferred ranges for very high density arrays (all numbers are per  $\text{cm}^2$ ) are from about 10,000,000 to about 2,000,000,000, with from about 100,000,000 to about 1,000,000,000 being preferred. High density arrays range from about 100,000  
30 to about 10,000,000, with from about 1,000,000 to about 5,000,000 being particularly preferred. Moderate density arrays range from about 10,000 to about 100,000 being particularly preferred, and from about 20,000 to about 50,000 being especially preferred. Low density arrays are generally less than 10,000, with from about 1,000 to about 5,000 being preferred. Very low density arrays are less than 1,000, with from about 10 to about 1000 being preferred, and from about 100 to about 500 being  
35 particularly preferred. In some embodiments, the compositions of the invention may not be in array



format; that is, for some embodiments, compositions comprising a single bioactive agent may be made as well. In addition, in some arrays, multiple substrates may be used, either of different or identical compositions. Thus for example, large arrays may comprise a plurality of smaller substrates.

5 In addition, one advantage of the present compositions is that particularly through the use of fiber optic technology, extremely high density arrays can be made. Thus for example, because beads of 200  $\mu\text{m}$  or less (with beads of 200 nm possible) can be used, and very small fibers are known, it is possible to have as many as 40,000 - 50,000 or more (in some instances, 1 million) different fibers and beads in a 1  $\text{mm}^2$  fiber optic bundle, with densities of greater than 15,000,000 individual beads and fibers (again, in some instances as many as 25-50 million) per 0.5  $\text{cm}^2$  obtainable.

10 The arrays comprise a substrate to which the beads are associated. By "substrate" or "solid support" or other grammatical equivalents herein is meant any material that can be modified to contain discrete individual sites appropriate for the attachment or association of beads and is amenable to at least one detection method. As will be appreciated by those in the art, the number of possible substrates is very large. Possible substrates include, but are not limited to, glass and modified or functionalized glass,  
15 plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, Teflon®, etc.), polysaccharides, nylon or nitrocellulose, resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses, plastics, optical fiber bundles, and a variety of other polymers. In general, the substrates allow optical detection and do not themselves appreciably fluoresce.

20 Generally the substrate is flat (planar), although as will be appreciated by those in the art, other configurations of substrates may be used as well; for example, three dimensional configurations can be used, for example by embedding the beads in a porous block of plastic that allows sample access to the beads and using a confocal microscope for detection. Similarly, the beads may be placed on the inside surface of a tube, for flow-through sample analysis to minimize sample volume. Preferred  
25 substrates include optical fiber bundles as discussed below, and flat planar substrates such as glass, polystyrene and other plastics and acrylics.

In a preferred embodiment, the substrate is an optical fiber bundle or array, as is generally described in U.S.S.N.s 08/944,850 and 08/519,062, PCT US98/05025, and PCT US98/05,033, all of which are expressly incorporated herein by reference. Preferred embodiments utilize preformed unitary fiber  
30 optic arrays. By "preformed unitary fiber optic array" herein is meant an array of discrete individual fiber optic strands that are co-axially disposed and joined along their lengths. The fiber strands are generally individually clad. However, one thing that distinguishes a preformed unitary array from other fiber optic formats is that the fibers are not individually physically manipulatable; that is, one strand generally cannot be physically separated at any point along its length from another fiber strand.

At least one surface of the substrate is modified to contain discrete, individual sites for later association of microspheres. These sites may comprise physically altered sites, i.e. physical configurations such as wells or small depressions in the substrate that can retain the beads, such that a microsphere can rest in the well, or the use of other forces (magnetic or compressive), or chemically altered or active sites, such as chemically functionalized sites, electrostatically altered sites, hydrophobically/ hydrophilically functionalized sites, spots of adhesive, etc.

The sites may be a pattern, i.e. a regular design or configuration, or randomly distributed. A preferred embodiment utilizes a regular pattern of sites such that the sites may be addressed in the X-Y coordinate plane. "Pattern" in this sense includes a repeating unit cell, preferably one that allows a high density of beads on the substrate. However, it should be noted that these sites may not be discrete sites. That is, it is possible to use a uniform surface of adhesive or chemical functionalities, for example, that allows the attachment of beads at any position. That is, the surface of the substrate is modified to allow attachment of the microspheres at individual sites, whether or not those sites are contiguous or non-contiguous with other sites. Thus, the surface of the substrate may be modified such that discrete sites are formed that can only have a single associated bead, or alternatively, the surface of the substrate is modified and beads may go down anywhere, but they end up at discrete sites.

In a preferred embodiment, the surface of the substrate is modified to contain wells, i.e. depressions in the surface of the substrate. This may be done as is generally known in the art using a variety of techniques, including, but not limited to, photolithography, stamping techniques, laser ablation, molding techniques and microetching techniques. As will be appreciated by those in the art, the technique used will depend on the composition and shape of the substrate. Thus, for example, for plastic substrates, the substrate may be molded or stamped during manufacture to produce the wells; fiber optic bundles may be chemically etched.

In a preferred embodiment, physical alterations are made in a surface of the substrate to produce the sites. In a preferred embodiment, the substrate is a fiber optic bundle and the surface of the substrate is a terminal end of the fiber bundle, as is generally described in 08/818,199 and 09/151,877, both of which are hereby expressly incorporated by reference. In this embodiment, wells are made in a terminal or distal end of a fiber optic bundle comprising individual fibers. In this embodiment, the cores of the individual fibers are etched, with respect to the cladding, such that small wells or depressions are formed at one end of the fibers. The methods used will depend on the composition of the substrate, with chemical etching being preferred for fiber optic bundles, for example using ammonium fluoride and hydrofluoric acid. The required depth of the wells will depend, in part, on the size of the beads to be added to the wells.

Generally in this embodiment, the microspheres are non-covalently associated in the wells, although the wells may additionally be chemically functionalized as is generally described below, cross-linking agents may be used, or a physical barrier may be used, i.e. a film or membrane over the beads.

5 In a preferred embodiment, the beads are attached non-covalently through non-specific interactions such as Van der Waals forces.

10 In a preferred embodiment, the surface of the substrate is modified to contain chemically modified sites that can be used to attach, either covalently or non-covalently, the microspheres of the invention to the discrete sites or locations on the substrate. "Chemically modified sites" in this context includes, but is not limited to, the addition of a pattern of chemical functional groups including amino groups, carboxy groups, oxo groups and thiol groups, that can be used to covalently attach microspheres, which generally also contain corresponding reactive functional groups; the addition of a pattern of adhesive that can be used to bind the microspheres (either by prior chemical functionalization for the addition of the adhesive or direct addition of the adhesive); the addition of a pattern of charged groups (similar to the chemical functionalities) for the electrostatic attachment of the microspheres, i.e. when 15 the microspheres comprise charged groups opposite to the sites; the addition of a pattern of chemical functional groups that renders the sites differentially hydrophobic or hydrophilic, such that the addition of similarly hydrophobic or hydrophilic microspheres under suitable experimental conditions will result in association of the microspheres to the sites on the basis of hydroaffinity. For example, the use of hydrophobic sites with hydrophobic beads, in an aqueous system, drives the association of the beads preferentially onto the sites. As outlined above, "pattern" in this sense includes the use of a uniform treatment of the surface to allow attachment of the beads at discrete sites, as well as treatment of the surface resulting in discrete sites. As will be appreciated by those in the art, this may be accomplished in a variety of ways.

20 The compositions of the invention further comprise a population of microspheres. By "population" herein is meant a plurality of beads as outlined above for arrays. Within the population are separate subpopulations, which can be a single microsphere or multiple identical microspheres. That is, in some embodiments, as is more fully outlined below, the array may contain only a single bead for each bioactive agent; preferred embodiments utilize a plurality of beads of each type.

30 By "microspheres" or "beads" or "particles" or grammatical equivalents herein is meant small discrete particles. The composition of the beads will vary, depending on the class of bioactive agent and the method of synthesis. Suitable bead compositions include those used in peptide, nucleic acid and organic moiety synthesis, including, but not limited to, plastics, ceramics, glass, polystyrene, methylstyrene, acrylic polymers including polyacrylimide, paramagnetic materials, thoria sol, carbon graphited, titanium dioxide, latex or cross-linked dextrans such as Sepharose, cellulose, nylon, cross-

linked micelles and teflon may all be used. "Microsphere Detection Guide" from Bangs Laboratories, Fishers IN is a helpful guide.

5 In a preferred embodiment, the beads are porous. That is, the interior of the bead is solvent accessible, with beads that are up to roughly 80% solvent (e.g. water) being preferred. Preferred embodiments utilize beads that have large internal surface areas as compared to the external surface of the bead. In addition, the pores are generally large enough to easily accomodate facile diffusion of biological molecules, particularly proteins and nucleic acids. Porous beads are generally made as is known in the art and generally result from differences in cross-linking and solvent conditions during formation.

10 The beads need not be spherical; irregular particles may be used. The bead sizes range from nanometers, i.e. 100 nm, to millimeters, i.e. 1 mm, with beads from about 0.2 micron to about 200 microns being preferred, and from about 0.5 to about 5 micron being particularly preferred, although in some embodiments smaller beads may be used.

15 It should be noted that a key component of the invention is the use of a substrate/bead pairing that allows the association or attachment of the beads at discrete sites on the surface of the substrate, such that the beads do not move during the course of the assay.

20 Each microsphere comprises at least one bioactive agent, although as will be appreciated by those in the art, there may be some microspheres which do not contain a bioactive agent, depending on the synthetic methods. Similarly, although not preferred, are beads comprising more than one bioactive agent. By "candidate bioactive agent" or "bioactive agent" or "chemical functionality" or "binding ligand" herein is meant as used herein describes any molecule, e.g., protein, oligopeptide, small organic molecule, coordination complex, polysaccharide, polynucleotide, etc. that can be attached to the microspheres of the invention. It should be understood that the compositions of the invention have two primary uses. In a preferred embodiment, as is more fully outlined below, the compositions are  
25 used to detect the binding of a candidate bioactive agent to a particular target analyte; for example, for screening of enzyme inhibitors or protein-protein interactions.

30 Bioactive agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 100 and less than about 2,500 daltons. Bioactive agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The bioactive agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Bioactive agents are also found among biomolecules including peptides, nucleic acids, saccharides, fatty acids, steroids, purines, pyrimidines,

derivatives, structural analogs or combinations thereof. Particularly preferred are nucleic acids and proteins.

5 Bioactive agents can be obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized  
oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant  
and animal extracts are available or readily produced. Additionally, natural or synthetically produced  
libraries and compounds are readily modified through conventional chemical, physical and  
10 biochemical means. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification and/or amidification to produce structural analogs.

In a preferred embodiment, the bioactive agents are proteins. By "protein" herein is meant at least two covalently attached amino acids, which includes proteins, polypeptides, oligopeptides and peptides.  
The protein may be made up of naturally occurring amino acids and peptide bonds, or synthetic  
15 peptidomimetic structures. Thus "amino acid", or "peptide residue", as used herein means both naturally occurring and synthetic amino acids. For example, homo-phenylalanine, citrulline and norleucine are considered amino acids for the purposes of the invention. The side chains may be in either the (R) or the (S) configuration. In the preferred embodiment, the amino acids are in the (S) or L-configuration. If non-naturally occurring side chains are used, non-amino acid substituents may be  
20 used, for example to prevent or retard in vivo degradations.

In one preferred embodiment, the bioactive agents are naturally occurring proteins or fragments of naturally occurring proteins. Thus, for example, cellular extracts containing proteins, or random or directed digests of proteinaceous cellular extracts, may be used. In this way libraries of procaryotic and eukaryotic proteins may be made for screening in the systems described herein. Particularly  
25 preferred in this embodiment are libraries of bacterial, fungal, viral, and mammalian proteins, with the latter being preferred, and human proteins being especially preferred.

In a preferred embodiment, the bioactive agents are peptides of from about 5 to about 30 amino acids, with from about 5 to about 20 amino acids being preferred, and from about 7 to about 15 being particularly preferred. The peptides may be digests of naturally occurring proteins as is outlined  
30 above, random peptides, or "biased" random peptides. By "randomized" or "grammatical equivalents" herein is meant that each nucleic acid and peptide consists of essentially random nucleotides and amino acids, respectively. Since generally these random peptides (or nucleic acids, discussed below) are chemically synthesized, they may incorporate any nucleotide or amino acid at any position. The synthetic process can be designed to generate randomized proteins or nucleic acids, to allow the

formation of all or most of the possible combinations over the length of the sequence, thus forming a library of randomized bioactive proteinaceous agents.

5 In a preferred embodiment, a library of bioactive agents are used. The library should provide a sufficiently structurally diverse population of bioactive agents to effect a probabilistically sufficient range of binding to target analytes. Accordingly, an interaction library must be large enough so that at least one of its members will have a structure that gives it affinity for the target analyte. Although it is difficult to gauge the required absolute size of an interaction library, nature provides a hint with the immune response: a diversity of  $10^7$ - $10^8$  different antibodies provides at least one combination with sufficient affinity to interact with most potential antigens faced by an organism. Published in vitro  
10 selection techniques have also shown that a library size of  $10^7$  to  $10^8$  is sufficient to find structures with affinity for the target. Thus, in a preferred embodiment, at least  $10^6$ , preferably at least  $10^7$ , more preferably at least  $10^8$  and most preferably at least  $10^9$  different bioactive agents are simultaneously analyzed in the subject methods. Preferred methods maximize library size and diversity.

15 In a preferred embodiment, the library is fully randomized, with no sequence preferences or constants at any position. In a preferred embodiment, the library is biased. That is, some positions within the sequence are either held constant, or are selected from a limited number of possibilities. For example, in a preferred embodiment, the nucleotides or amino acid residues are randomized within a defined class, for example, of hydrophobic amino acids, hydrophilic residues, sterically biased (either small or large) residues, towards the creation of cysteines, for cross-linking, prolines for SH-3  
20 domains, serines, threonines, tyrosines or histidines for phosphorylation sites, etc., or to purines, etc.

In a preferred embodiment, the bioactive agents are nucleic acids (generally called "probe nucleic acids" or "candidate probes" herein). By "nucleic acid" or "oligonucleotide" or grammatical equivalents herein means at least two nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below,  
25 nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramidate (Beaucage, et al., Tetrahedron, 49(10):1925 (1993) and references therein; Letsinger, J. Org. Chem., 35:3800 (1970); Sprinzl, et al., Eur. J. Biochem., 81:579 (1977); Letsinger, et al., Nucl. Acids Res., 14:3487 (1986); Sawai, et al., Chem. Lett., 805 (1984), Letsinger, et al., J. Am. Chem. Soc., 110:4470 (1988); and Pauwels, et al., Chemica Scripta, 26:141 (1986)), phosphorothioate (Mag,  
30 et al., Nucleic Acids Res., 19:1437 (1991); and U.S. Patent No. 5,644,048), phosphorodithioate (Briu, et al., J. Am. Chem. Soc., 111:2321 (1989)), O-methylphosphoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, J. Am. Chem. Soc., 114:1895 (1992); Meier, et al., Chem. Int. Ed. Engl., 31:1008 (1992); Nielsen, Nature, 365:566 (1993); Carlsson, et al., Nature, 380:207  
35 (1996), all of which are incorporated by reference)). Other analog nucleic acids include those with positive backbones (Denpcy, et al., Proc. Natl. Acad. Sci. USA, 92:6097 (1995)); non-ionic backbones

(U.S. Patent Nos. 5,386,023; 5,637,684; 5,602,240; 5,216,141; and 4,469,863; Kiedrowshi, et al., Angew. Chem. Intl. Ed. English, 30:423 (1991); Letsinger, et al., J. Am. Chem. Soc., 110:4470 (1988); Letsinger, et al., Nucleosides & Nucleotides, 13:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker, et al., Bioorganic & Medicinal Chem. Lett., 4:395 (1994); Jeffs, et al., J. Biomolecular NMR, 34:17 (1994); Tetrahedron Lett., 37:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins, et al., Chem. Soc. Rev., (1995) pp. 169-176). Several nucleic acid analogs are described in Rawls, C & E News, June 2, 1997, page 35. All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of additional moieties such as labels, or to increase the stability and half-life of such molecules in physiological environments; for example, PNA is particularly preferred. In addition, mixtures of naturally occurring nucleic acids and analogs can be made. Alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occurring nucleic acids and analogs may be made. The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid contains any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthanine, hypoxanthanine, isocytosine, isoguanine, and base analogs such as nitropyrrole and nitroindole, etc.

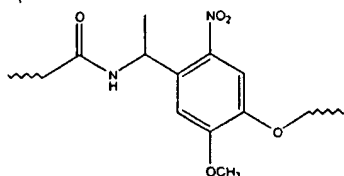
As described above generally for proteins, nucleic acid bioactive agents may be naturally occurring nucleic acids, random nucleic acids, or "biased" random nucleic acids. For example, digests of procaryotic or eukaryotic genomes may be used as is outlined above for proteins.

In a preferred embodiment, as is more fully described below, the candidate agent comprises a fluorophore, that will exhibit a change in its fluorescent properties upon interaction (binding) with a target analyte.

In a preferred embodiment, the candidate agent is linked, preferably covalently, to the bead with a scissile linkage, such that the candidate agent can be cleaved from the bead prior to or during the assay as is more fully described below. By "scissile" or "cleavable" linkage herein is meant a linkage that is cleavable under assay conditions; that is, the candidate bioactive agent can be separated from the bead, resulting in a molecule that is essentially free to diffuse throughout the interior and exterior of the bead. In general, covalent linkages that are scissile are preferred, although non-covalent scissile linkages can also be used.

In a preferred embodiment, the scissile linkage is a photocleavable linkage, that is, a linkage that will be cleaved by exposure to light, particularly specific wavelengths of light. Thus, for example, photocleavable linkers such as ortho-nitrobenzyl groups,  $\alpha$ -methylphenacyl ester, etc. may be used. See for example *A Practical Guide to Combinatorial Chemistry*, Eds. A. W. Czarnik and S.H. DeWitt, American Chemical Society, Washington D.C., 1997, hereby incorporated by reference in its entirety.

5 A preferred photocleavable linker is shown below:



In a preferred embodiment, all the different candidate agents are attached to the beads using a single type of photocleavable linker, such that a single uniform application of light at the active wavelength will cause all of the agents to be released simultaneously. Alternatively, different photocleavable linkers may be used, for example to allow sequential monitoring of reactions, or "batch" analysis.

In a preferred embodiment, the scissile linkage may be an enzymatically cleavable linkage. Depending on the composition of the candidate agent and the desired linkage, there are any number of suitable cleavage enzymes with known cleavage sites that can be used, including, but not limited to, hydrolases such as proteases, carbohydrases and lipases. For example, cleavable linkers may be used to attach the agents to the beads; for example, a protease may be used when the linker is a protein, a carbohydrase may be used when the linker is a carbohydrate, a lipase may be used when the linker is a lipid, etc.

As will be appreciated by those in the art, there are a wide variety of enzymatically cleavable sites that may be used to attach the agents to the beads. For example, sequences that are recognized and  
20 cleaved by a protease or cleaved after exposure to certain chemicals are considered cleavable linkers. For example, cleavable linkers include, but are not limited to, the prosequence of bovine chymosin, the prosequence of subtilisin, prosequences of retroviral proteases including human immunodeficiency virus protease and sequences recognized and cleaved by trypsin (EP 578472, Takasuga et al., J. Biochem. 112(5):652 (1992)) factor X<sub>a</sub> (Gardella et al., J. Biol. Chem.  
25 265(26):15854 (1990), WO 900637C), collagenase (J03280893, Tajima et al., J. Ferment. Bioeng. 72(5):362 (1991), WO 9006370), clostripain (EP 578472), subtilisin (including mutant H64A subtilisin, Forsberg et al., J. Protein Chem. 10(5):517 (1991), chymosin, yeast KEX2 protease (Bourbonnais et al., J. Bio. Chem. 263(30):15342 (1988), thrombin (Forsberg et al., supra; Abath et al., BioTechniques 10(2):178 (1991)), *Staphylococcus aureus* V8 protease or similar endoproteinase-Glu-C to cleave  
30 after Glu residues (EP 578472, Ishizaki et al., Appl. Microbiol. Biotechnol. 36(4):483 (1992)), cleavage by Nla proteainase of tobacco etch virus (Parks et al., Anal. Biochem. 216(2):413 (1994)), endoproteinase-Lys-C (U.S. Patent No. 4,414,332) and endoproteinase-Asp-N, *Neisseria* type 2 IgA



protease (Pohlner et al., *Bio/Technology* 10(7):799-804 (1992)), soluble yeast endoproteinase yscF (EP 467839), chymotrypsin (Altman et al., *Protein Eng.* 4(5):593 (1991)), enteropeptidase (WO 9006370), lysostaphin, a polyglycine specific endoproteinase (EP 316748), and the like. See e.g. Marston, F.A.O. (1986) *Biol. Chem. J.* 240, 1-12.

5 Other types of labile linkages may be used, as will be appreciated by those in the art. For example, the scissile linkage may be an acid labile linkage, and other cleavable linkages. Particular amino acid sites that serve as chemical cleavage sites include, but are not limited to, methionine for cleavage by cyanogen bromide (Shen, *PNAS USA* 81:4627 (1984); Kempe et al., *Gene* 39:239 (1985); Kuliopulos et al., *J. Am. Chem. Soc.* 116:4599 (1994); Moks et al., *Bio/Technology* 5:379 (1987); Ray et al.,  
10 *Bio/Technology* 11:64 (1993)), acid cleavage of an Asp-Pro bond (Wingender et al., *J. Biol. Chem.* 264(8):4367 (1989); Gram et al., *Bio/Technology* 12:1017 (1994)), and hydroxylamine cleavage at an Asn-Gly bond (Moks supra). Thus, suitable linkers include, but are not limited to, p-hydroxymethylbenzoic acid, 4-hydroxymethylphenylacetic acid, benzhydrylamino, allyl, hydroxyl-crotonyl-aminomethyl, 3-nitro-4-hydroxymethylbenzoic acid, p-nitrobenzydrylamine and 4-[4,4'-bis(methylsulfinyl)-2-oxy-benzhydrylamino]butyric acid.  
15

In one embodiment, the candidate agent is linked to the bead via a non-cleavable flexible linker. That is, the cleavage may not be required, if the linker attaching the agent to the bead is flexible enough to allow interaction with a target. Thus, linkers such as alkyl groups, including substituted alkyl groups and heteroalkyl groups, may be used.

20 In a preferred embodiment, each bead comprises a single type of bioactive agent, although a plurality of individual bioactive agents are preferably attached to each bead. Similarly, preferred embodiments utilize more than one microsphere containing a unique bioactive agent; that is, there is redundancy built into the system by the use of subpopulations of microspheres, each microsphere in the subpopulation containing the same bioactive agent.

25 In addition, as is more fully outlined below, the candidate agents may further comprise detectable labels that will alter their characteristics upon interaction (binding) with a target analyte.

As will be appreciated by those in the art, the bioactive agents may either be synthesized directly on the beads, or they may be made and then attached after synthesis.

30 In a preferred embodiment, the bioactive agents are synthesized directly on the beads. As is known in the art, many classes of chemical compounds are currently synthesized on solid supports, such as peptides, organic moieties, and nucleic acids. It is a relatively straightforward matter to adjust the current synthetic techniques to use porous beads.

In a preferred embodiment, the bioactive agents are synthesized first, and then covalently attached to the beads. As will be appreciated by those in the art, this will be done depending on the composition of the bioactive agents, the scissile linkage and the beads. The functionalization of solid support surfaces such as certain polymers with chemically reactive groups such as thiols, amines, carboxyls, etc. is generally known in the art. Accordingly, "blank" microspheres may be used that have surface chemistries that facilitate the attachment of the desired functionality by the user. Some examples of these surface chemistries for blank microspheres include, but are not limited to, amino groups including aliphatic and aromatic amines, carboxylic acids, aldehydes, amides, chloromethyl groups, hydrazide, hydroxyl groups, sulfonates and sulfates.

These functional groups can be used to add any number of different candidate agents to the beads, generally using known chemistries. For example, candidate agents containing carbohydrates may be attached to an amino-functionalized support; the aldehyde of the carbohydrate is made using standard techniques, and then the aldehyde is reacted with an amino group on the surface. In an alternative embodiment, a sulfhydryl linker may be used. There are a number of sulfhydryl reactive linkers known in the art such as SPDP, maleimides,  $\alpha$ -haloacetyls, and pyridyl disulfides (see for example the 1994 Pierce Chemical Company catalog, technical section on cross-linkers, pages 155-200, incorporated herein by reference) which can be used to attach cysteine containing proteinaceous agents to the support. Alternatively, an amino group on the candidate agent may be used for attachment to an amino group on the surface. For example, a large number of stable bifunctional groups are well known in the art, including homobifunctional and heterobifunctional linkers (see Pierce Catalog and Handbook, pages 155-200). In an additional embodiment, carboxyl groups (either from the surface or from the candidate agent) may be derivatized using well known linkers (see the Pierce catalog). For example, carbodiimides activate carboxyl groups for attack by good nucleophiles such as amines (see Torchilin et al., *Critical Rev. Therapeutic Drug Carrier Systems*, 7(4):275-308 (1991), expressly incorporated herein). Proteinaceous candidate agents may also be attached using other techniques known in the art, for example for the attachment of antibodies to polymers; see Slinkin et al., *Bioconj. Chem.* 2:342-348 (1991); Torchilin et al., *supra*; Trubetskoy et al., *Bioconj. Chem.* 3:323-327 (1992); King et al., *Cancer Res.* 54:6176-6185 (1994); and Wilbur et al., *Bioconjugate Chem.* 5:220-235 (1994), all of which are hereby expressly incorporated by reference). It should be understood that the candidate agents may be attached in a variety of ways, including those listed above. What is important is that manner of attachment allows the incorporation of a scissile linkage and does not significantly alter the functionality of the candidate agent.

In some embodiments, there may be two species linked to the beads, using the techniques outlined above. Thus, for example, a bioactive agent being tested as an inhibitor may be synthesized on the beads, and a known enzyme substrate may be attached subsequently, using either the same cleavable linkage or a different one. Upon exposure to the cleavage agent, both molecules are released, allowing competitive assay analysis. Thus, some embodiments utilize beads comprising

more than one assay component, all of which can be either synthesized on the bead, added to the bead after synthesis, or both. In addition, there may be one species linked to the bead and a second species is diffused in prior to the assay.

5 The present invention further provides a unique high-throughput screening advantage: the ability to vary the concentration of the agent in the assay from bead to bead, thus allowing concentration effects to be monitored simultaneously. This may be done in a number of ways, as will be appreciated by those in the art. As outlined above, this provides a number of significant advantages, including the ability to monitor kinetic parameters and thus avoid resynthesis of the agents and reassaying.

10 In a preferred embodiment, the number of "active sites" for chemical synthesis on and inside the beads is varied. That is, as is well known in the art, the number or density of synthetic sites on a surface such as a bead can be varied. This will allow the concentration of the candidate agents in an assay to vary from bead to bead. That is, since the amount of chemical synthesis will be determined by the number of active sites within the bead, the amount of agent synthesized can be varied. Since  
15 the assay "volume" is effectively the same from bead to bead, upon release of the agent from the bead prior to or during the assay, the effective concentration of the agent in the assay will be different for the different beads. By coding the beads according to the number of active sites on the bead, as is more fully outlined below, the effects of concentration differences can be observed.

20 In a preferred embodiment, the number of active sites is not altered, but the functionality of the active sites is varied by adding capping agents and/or protecting agents. That is, a set number of active sites may be exposed to different concentrations of capping agents, resulting in different amounts of capping or inactivation of the sites. This may be done with protecting groups as well, where a standard synthetic protecting group is added to some active sites, followed by capping. Alternatively, mixtures of capping agent and protecting agents are made in different ratios, resulting in different amounts of available active groups for addition of the agents.

25 In a preferred embodiment, this concentration difference can be generated not by altering the number of active sites for synthesis, but by altering the composition of the cleavable linker such that the candidate agents are released at different rates. Thus for example, a first cleavable linker that results in fast cleavage is used for a first concentration of agent; a second cleavable linker that is slower is used for a second (lower) concentration of agent, etc.

30 In a preferred embodiment, this concentration difference can be generated by altering the amount of the cleavage agent introduced to the beads. For example, the same photocleavable linker may be used, but the amount of light, i.e. the number of photons, introduced to different beads is varied, resulting in different amounts of release. This may be done by using different "masking" techniques, as is well known in the art. This may provide the additional benefit of allowing the use of fewer codes,

in that by using masks, spatial information about the location of different concentrations of agents can be acquired.

5 In a preferred embodiment, the microspheres further comprise an identifier moiety. As is more fully outlined below, the microspheres may be randomly associated with a substrate, such that each bead is localized at a discrete yet random site on the substrate. That is, the microspheres, each carrying different chemical functionalities are distributed on a substrate comprising a patterned surface of discrete sites that can bind the individual microspheres. The beads are generally put onto the substrate randomly, and thus several different methodologies can be used to "decode" the arrays, i.e. correlate the identity of a candidate agent with a location on the array. This is generally done using identifier moieties, which can utilize several different mechanisms to result in decoding of the random array. In one embodiment, the identifier moieties are unique optical signatures that are incorporated into the beads, for example fluorescent dyes, that can be used to optically identify the chemical functionality on any particular bead; other types of non-optical identifier moieties may also be used, as is described below. Alternatively, the identifier moiety may be an identifier binding ligand (IBL), to which a decoding binding ligand (DBL) will bind, allowing identification of the candidate agent bound to the bead. All of these methods allow the synthesis of the candidate agents (i.e. compounds such as nucleic acids and antibodies) to be divorced from their placement on an array, i.e. the candidate agents may be synthesized on the beads, and then the beads are randomly distributed on a patterned surface. Since the beads are first coded with an identifier moiety, this means that the array can later be "decoded", i.e. after the array is made, a correlation of the location of an individual site on the array with the bead or candidate agent at that particular site can be made. In addition, decoding may be accomplished by positional decoding, for example by either targeting the placement of beads (for example by using photoactivatable or photocleavable moieties to allow the selective addition of beads to particular locations), or by using either sub-bundles or selective loading of the sites, as are more fully outlined below. As is also discussed below, decoding may occur either prior to or after addition of a target analyte. The development of the subsequent decoding technology means that the beads may be randomly distributed on the array, a fast and inexpensive process as compared to either the in situ synthesis or spotting techniques of the prior art. These methods are generally outlined in PCT US98/05025 and U.S.S.N.s 08/818,199 and 09/151,877, all of which are expressly incorporated herein by reference.

30 In addition, the identifier moieties are not detached or removed from the beads for identification. That is, correlation of the identifier moiety and the location of the bead generally happens in situ, with the bead still on the array. This is in contrast to the "mass tags" types of systems described in U.S. patent No. 5,836,083; thus, the identifier moieties are not removable gas-chromatograph or mass- spectroscopy tags as are known in the art. However, in some embodiments, after decoding, it may be possible to remove the identifier moieties so that they do not interfere with any subsequent reaction.

Accordingly, in a preferred embodiment, the identifier moiety is an optical signature that can be used to identify the attached bioactive agent. That is, in this embodiment, each subpopulation of microspheres comprises a unique optical signature or optical tag that can be used to identify the unique bioactive agent of that subpopulation of microspheres; a bead comprising the unique optical signature may be distinguished from beads at other locations with different optical signatures. As is outlined herein, each bioactive agent will have an associated unique optical signature such that any microspheres comprising that bioactive agent will be identifiable on the basis of the signature. As is more fully outlined below, it is possible to reuse or duplicate optical signatures within an array, for example, when another level of identification is used, for example when beads of different sizes are used, or when the array is loaded sequentially with different batches of beads.

In a preferred embodiment, the optical signature is generally a mixture of reporter dyes, preferably fluorescent. By varying both the composition of the mixture (i.e. the ratio of one dye to another) and the concentration of the dye (leading to differences in signal intensity), matrices of unique tags may be generated. This may be done by covalently attaching the dyes to the surface of the beads, or alternatively, by entrapping the dye within the bead. The dyes may be chromophores or phosphors but are preferably fluorescent dyes, which due to their strong signals provide a good signal-to-noise ratio for decoding. Suitable dyes for use in the invention include, but are not limited to, fluorescent lanthanide complexes, including those of Europium and Terbium, fluorescein, rhodamine, tetramethylrhodamine, eosin, erythrosin, coumarin, methyl-coumarins, pyrene, Malacite green, stilbene, Lucifer Yellow, Cascade Blue™, Texas Red, and others described in the 6th Edition of Molecular Probes Handbook by Richard P. Haugland, hereby expressly incorporated by reference.

In a preferred embodiment, the encoding can be accomplished in a ratio of at least two dyes, although more encoding dimensions may be added in the size of the beads, for example. In addition, the labels are distinguishable from one another; thus two different labels may comprise different molecules (i.e. two different fluors) or, alternatively, one label at two different concentrations or intensity.

In a preferred embodiment, the dyes are covalently attached to the surface of the beads. This may be done as is generally outlined for the attachment of the bioactive agents, using functional groups on the surface of the beads. As will be appreciated by those in the art, these attachments are done to minimize the effect on the dye.

In a preferred embodiment, the dyes are non-covalently associated with the beads, generally by entrapping the dyes in the bead matrix or pores of the beads. Additionally, encoding in the ratios of the two or more dyes, rather than single dye concentrations, is preferred since it provides insensitivity to the intensity of light used to interrogate the reporter dye's signature and detector sensitivity.

In a preferred embodiment, the identifier moiety is an identifier binding ligand. By "identifier binding ligands" or "IBLs" herein is meant a compound that will specifically bind a corresponding decoder binding ligand (DBL) to facilitate the elucidation of the identity of the bioactive agent attached to the bead. That is, the IBL and the corresponding DBL form a binding partner pair. By "specifically bind" herein is meant that the IBL binds its DBL with specificity sufficient to differentiate between the corresponding DBL and other DBLs (that is, DBLs for other IBLs), or other components or contaminants of the system. The binding should be sufficient to remain bound under the conditions of the decoding step, including wash steps to remove non-specific binding. In some embodiments, for example when the IBLs and corresponding DBLs are proteins or nucleic acids, the dissociation constants of the IBL to its DBL will be less than about  $10^{-4}$ - $10^{-6}$  M<sup>-1</sup>, with less than about  $10^{-5}$  to  $10^{-9}$  M<sup>-1</sup> being preferred and less than about  $10^{-7}$  -  $10^{-9}$  M<sup>-1</sup> being particularly preferred.

IBL-DBL binding pairs are known or can be readily found using known techniques. For example, when the IBL is a protein, the DBLs include proteins (particularly including antibodies or fragments thereof (Fabs, etc.)) or small molecules, or vice versa (the IBL is an antibody and the DBL is a protein). Metal ion- metal ion ligands or chelators pairs are also useful. Antigen-antibody pairs, enzymes and substrates or inhibitors, other protein-protein interacting pairs, receptor-ligands, complementary nucleic acids, and carbohydrates and their binding partners are also suitable binding pairs. Nucleic acid - nucleic acid binding proteins pairs are also useful. Similarly, as is generally described in U.S. Patents 5,270,163, 5,475,096, 5,567,588, 5,595,877, 5,637,459, 5,683,867, 5,705,337, and related patents, hereby incorporated by reference, nucleic acid "aptamers" can be developed for binding to virtually any target; such a aptamer-target pair can be used as the IBL-DBL pair. Similarly, there is a wide body of literature relating to the development of binding pairs based on combinatorial chemistry methods.

As for the agent-target binding, a preferred embodiment utilizes IBLs that are molecules whose color or luminescence properties change in the presence of a selectively-binding DBL. For example, the IBL may be a fluorescent pH indicator whose emission intensity changes with pH. Similarly, the IBL may be a fluorescent ion indicator, whose emission properties change with ion concentration. Alternatively, the IBL is a molecule whose color or luminescence properties change in the presence of various solvents. For example, the IBL may be a fluorescent molecule such as an ethidium salt whose fluorescence intensity increases in hydrophobic environments. Similarly, the IBL may be a derivative of fluorescein whose color changes between aqueous and nonpolar solvents.

In one embodiment, the DBL may be attached to a bead, i.e. a "decoder bead", that may carry a label such as a fluorophore.

In a preferred embodiment, the IBL-DBL pair comprise substantially complementary single-stranded nucleic acids. In this embodiment, the binding ligands can be referred to as "identifier probes" and

"decoder probes". Generally, the identifier and decoder probes range from about 4 basepairs in length to about 1000, with from about 6 to about 100 being preferred, and from about 8 to about 40 being particularly preferred. What is important is that the probes are long enough to be specific, i.e. to distinguish between different IBL-DBL pairs, yet short enough to allow both a) dissociation, if necessary, under suitable experimental conditions, and b) efficient hybridization.

In general, probes of the present invention are designed to be complementary to a target sequence (i.e. a DBL), such that hybridization of the target and the probes of the present invention occurs. This complementarity need not be perfect; there may be any number of base pair mismatches that will interfere with hybridization between the target sequence and the single stranded nucleic acid probes of the present invention. However, if the number of mutations is so great that no hybridization can occur under even the least stringent of hybridization conditions, the sequence is not a complementary target sequence. Thus, by "substantially complementary" herein is meant that the probes are sufficiently complementary to the target sequences to hybridize under the selected reaction conditions. High stringency conditions are known in the art; see for example Maniatis et al., *Molecular Cloning: A Laboratory Manual*, 2d Edition, 1989, and *Short Protocols in Molecular Biology*, ed. Ausubel, et al., both of which are hereby incorporated by reference. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point ( $T_m$ ) for the specific sequence at a defined ionic strength pH. The  $T_m$  is the temperature (under defined ionic strength, pH and nucleic acid concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at  $T_m$ , 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g. 10 to 50 nucleotides) and at least about 60°C for long probes (e.g. greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. In another embodiment, less stringent hybridization conditions are used; for example, moderate or low stringency conditions may be used, as are known in the art; see Maniatis and Ausubel, *supra*, and Tijssen, *supra*.

Alternatively, the identifier moiety ("IM") need not necessarily bind to DBLs. That is, rather than elucidate the structure of the bioactive agent directly, the composition of the IMs may serve as the identifier. Thus, for example, a specific combination of IMs can serve to code the bead; antibodies to the individual IMs can then be used for decoding.

In a preferred embodiment, each subpopulation of beads comprises a plurality of different IBLs. By using a plurality of different IBLs to encode each bioactive agent, the number of possible unique codes is substantially increased. That is, by using one unique IBL per bioactive agent, the size of the array will be the number of unique IBLs (assuming no "reuse" occurs, as outlined below). However, by using a plurality of different IBLs per bead,  $n$ , the size of the array can be increased to  $2^n$ , when the presence or absence of each IBL is used as the indicator. For example, the assignment of 10 IBLs per bead generates a 10 bit binary code, where each bit can be designated as "1" (IBL is present) or "0" (IBL is absent). A 10 bit binary code has  $2^{10}$  possible variants. However, as is more fully discussed below, the size of the array may be further increased if another parameter is included such as concentration or intensity; thus for example, if two different concentrations of the IBL are used, then the array size increases as  $3^n$ . Thus, in this embodiment, each individual bioactive agent in the array is assigned a combination of IBLs, which can be added to the beads prior to the addition of the bioactive agent, after, or during the synthesis of the bioactive agent, i.e. simultaneous addition of IBLs and bioactive agent components.

Alternatively, when the bioactive agent is a polymer of different residues, i.e. when the bioactive agent is a protein or nucleic acid, the combination of different IBLs can be used to elucidate the sequence of the protein or nucleic acid.

Thus, for example, using two different IBLs (IBL1 and IBL2), the first position of a nucleic acid can be elucidated: for example, adenosine can be represented by the presence of both IBL1 and IBL2; thymidine can be represented by the presence of IBL1 but not IBL2, cytosine can be represented by the presence of IBL2 but not IBL1, and guanosine can be represented by the absence of both. The second position of the nucleic acid can be done in a similar manner using IBL3 and IBL4; thus, the presence of IBL1, IBL2, IBL3 and IBL4 gives a sequence of AA; IBL1, IBL2, and IBL3 shows the sequence AT; IBL1, IBL3 and IBL4 gives the sequence TA, etc. The third position utilizes IBL5 and IBL6, etc. In this way, the use of 20 different identifiers can yield a unique code for every possible 10-mer.

The system is similar for proteins but requires a larger number of different IBLs to identify each position, depending on the allowed diversity at each position. Thus for example, if every amino acid is allowed at every position, five different IBLs are required for each position. However, as outlined above, for example when using random peptides as the bioactive agents, there may be bias built into the system; not all amino acids may be present at all positions, and some positions may be preset; accordingly, it may be possible to utilize four different IBLs for each amino acid.

In this way, a sort of "bar code" for each sequence can be constructed; the presence or absence of each distinct IBL will allow the identification of each bioactive agent.



In addition, the use of different concentrations or densities of IBLs allows a "reuse" of sorts. If, for example, the bead comprising a first agent has a 1X concentration of IBL, and a second bead comprising a second agent has a 10X concentration of IBL, using sufficiently high (e.g. saturating) concentrations of the corresponding labelled DBL allows the user to distinguish between the two beads.

Accordingly, the beads of the invention comprise candidate bioactive agents and identifier moieties. In a preferred embodiment, the beads are distributed on the surface. In general, the methods of making the arrays and of decoding the arrays is done to maximize the number of different candidate agents that can be uniquely encoded. The compositions of the invention may be made in a variety of ways. In general, the arrays are made by adding a solution or slurry comprising the beads to a surface containing the sites for attachment of the beads. This may be done in a variety of buffers, including aqueous and organic solvents, and mixtures. The solvent can evaporate, and excess beads removed. Alternatively, the surface comprising the sites, particularly wells, can be dipped into a dry mixture of the beads, and the excess "tapped" off.

In a preferred embodiment, when non-covalent methods are used to associate the beads to the array, a novel method of loading the beads onto the array is used. This method comprises exposing the array to a solution of particles and then applying energy, e.g. agitating or vibrating the mixture. This results in an array comprising more tightly associated particles, as the agitation is done with sufficient energy to cause weakly-associated beads to fall off (or out, in the case of wells). These sites are then available to bind a different bead. In this way, beads that exhibit a high affinity for the sites are selected. Arrays made in this way have two main advantages as compared to a more static loading: first of all, a higher percentage of the sites can be filled easily, and secondly, the arrays thus loaded show a substantial decrease in bead loss during assays. Thus, in a preferred embodiment, these methods are used to generate arrays that have at least about 50% of the sites filled, with at least about 75% being preferred, and at least about 90% being particularly preferred. Similarly, arrays generated in this manner preferably lose less than about 20% of the beads during an assay, with less than about 10% being preferred and less than about 5% being particularly preferred.

In this embodiment, the substrate comprising the surface with the discrete sites is immersed into a solution comprising the particles. The surface may comprise wells, as is described herein, or other types of sites on a patterned surface such that there is a differential affinity for the sites. This differential affinity results in a competitive process, such that particles that will associate more tightly are selected. Preferably, the entire surface to be "loaded" with beads is in fluid contact with the solution. This solution is generally a slurry ranging from about 10,000:1 beads:solution (vol:vol) to 1:1. Generally, the solution can comprise any number of reagents, including aqueous buffers, organic solvents, salts, other reagent components, etc. In addition, the solution preferably comprises an

excess of beads; that is, there are more beads than sites on the array. Preferred embodiments utilize two-fold to billion-fold excess of beads.

5 The immersion can mimic the assay conditions; for example, if the array is to be "dipped" from above into a microtiter plate comprising samples, this configuration can be repeated for the loading, thus minimizing the beads that are likely to fall out due to gravity.

10 Once the surface has been immersed, the substrate, the solution, or both are subjected to a competitive process, whereby the particles with lower affinity can be disassociated from the substrate and replaced by particles exhibiting a higher affinity to the site. This competitive process is done by the introduction of energy, in the form of heat, sonication, stirring or mixing, vibrating or agitating the solution or substrate, or both.

15 A preferred embodiment utilizes agitation or vibration. In general, the amount of manipulation of the substrate is minimized to prevent damage to the array; thus, preferred embodiments utilize the agitation of the solution rather than the array, although either will work. As will be appreciated by those in the art, this agitation can take on any number of forms, with a preferred embodiment utilizing microtiter plates comprising bead solutions being agitated using microtiter plate shakers.

The agitation proceeds for a period of time sufficient to load the array to a desired fill. Depending on the size and concentration of the beads and the size of the array, this time may range from about 1 second to days, with from about 1 minute to about 24 hours being preferred.

20 It should be noted that not all sites of an array may comprise a bead; that is, there may be some sites of the substrate surface which are empty. In addition, there may be some sites that contain more than one bead, although this is generally not preferred.

In a preferred embodiment, the compositions of the invention are made in a composite array format, as is generally described in PCT US99/31022, hereby incorporated by reference in its entirety.

25 In one embodiment, the beads are not distributed on a surface, but are used in solution, with reaction monitoring and decoding occurring using a FACS, for example. In this embodiment, the beads are preferably put into a diffusion-retarding solvent, for example an organic solvent such as hexane, such that the reaction components do not freely diffuse out of the bead. High viscosity aqueous solvents can also be used.

30 In some embodiments, for example when chemical attachment is done, it is possible to attach the beads in a non-random or ordered way. For example, using photoactivatable attachment linkers or

photoactivatable adhesives or masks, selected sites on the array may be sequentially rendered suitable for attachment, such that defined populations of beads are laid down.

5 The arrays of the present invention are constructed such that information about the identity of the candidate agent is built into the array, such that the random deposition of the beads on the surface, for example in fiber wells, can be "decoded" to allow identification of the candidate agent at all positions. This may be done in a variety of ways, and either before, during or after the use of the array to detect target molecules.

Thus, after the array is made, it is "decoded" in order to identify the location of one or more of the bioactive agents, i.e. each subpopulation of beads, on the substrate surface.

10 In a preferred embodiment, a selective decoding system is used. In this case, only those microspheres exhibiting a change in the optical signal as a result of the binding of a target analyte are decoded. This is commonly done when the number of "hits", i.e. the number of sites to decode, is generally low. That is, the array is first scanned under experimental conditions in the absence of the target analytes. The sample containing the target analytes is added, and only those locations  
15 exhibiting a change in the optical signal are decoded. For example, the beads at either the positive or negative signal locations may be either selectively tagged or released from the array (for example through the use of photocleavable linkers generally different from those used to link the candidate agents to the beads), and subsequently sorted or enriched in a fluorescence-activated cell sorter (FACS). That is, either all the negative beads are released, and then the positive beads are either  
20 released or analyzed in situ, or alternatively all the positives are released and analyzed. Alternatively, the labels may comprise halogenated aromatic compounds, and detection of the label is done using for example gas chromatography, chemical tags, isotopic tags, or mass spectral tags.

As will be appreciated by those in the art, this may also be done in systems where the array is not decoded; i.e. there need not ever be a correlation of bead composition with location. In this  
25 embodiment, the beads are loaded on the array, and the assay is run. The "positives", i.e. those beads displaying a change in the optical signal as is more fully outlined below, are then "marked" to distinguish or separate them from the "negative" beads. This can be done in several ways, preferably using fiber optic arrays. In a preferred embodiment, each bead contains a fluorescent dye. After the assay and the identification of the "positives" or "active beads", light is shown down either only the  
30 positive fibers or only the negative fibers, generally in the presence of a light-activated reagent (typically dissolved oxygen). In the former case, all the active beads are photobleached. Thus, upon non-selective release of all the beads with subsequent sorting, for example using a fluorescence activated cell sorter (FACS) machine, the non-fluorescent active beads can be sorted from the fluorescent negative beads. Alternatively, when light is shown down the negative fibers, all the  
35 negatives are non-fluorescent and the the postives are fluorescent, and sorting can proceed. The

characterization of the attached bioactive agent may be done directly, for example using mass spectroscopy.

Alternatively, the identification may occur through the use of identifier moieties that need not necessarily bind to DBLs. That is, rather than elucidate the structure of the bioactive agent directly, the composition of the IMs may serve as the identifier. Thus, for example, a specific combination of IMs can serve to code the bead, and be used to identify the agent on the bead upon release from the bead followed by subsequent analysis, for example using a gas chromatograph or mass spectroscope.

Alternatively, rather than having each bead contain a fluorescent dye, each bead comprises a non-fluorescent precursor to a fluorescent dye. For example, using photocleavable protecting groups (again, generally different from the photocleavable linkers attaching the candidate agents, although in some embodiments they may be the same), such as certain ortho-nitrobenzyl groups, on a fluorescent molecule, photoactivation of the fluorochrome can be done. After the assay, light is shown down again either the "positive" or the "negative" fibers, to distinguish these populations. The illuminated precursors are then chemically converted to a fluorescent dye. All the beads are then released from the array, with sorting, to form populations of fluorescent and non-fluorescent beads (either the positives and the negatives or vice versa).

In an alternate preferred embodiment, the sites of attachment of the beads (for example the wells) include a photopolymerizable reagent, or the photopolymerizable agent is added to the assembled array. After the test assay is run, light is shown down again either the "positive" or the "negative" fibers, to distinguish these populations. As a result of the irradiation, either all the positives or all the negatives are polymerized and trapped or bound to the sites, while the other population of beads can be released from the array.

In a preferred embodiment, the location of every bioactive agent is determined using decoder binding ligands (DBLs). As outlined above, DBLs are binding ligands that will either bind to identifier binding ligands, if present, or to the bioactive agents themselves, preferably when the bioactive agent is a nucleic acid or protein.

In a preferred embodiment, as outlined above, the DBL binds to the IBL.

In a preferred embodiment, the bioactive agents are single-stranded nucleic acids and the DBL is a substantially complementary single-stranded nucleic acid that binds (hybridizes) to the bioactive agent, termed a decoder probe herein. A decoder probe that is substantially complementary to each candidate probe is made and used to decode the array. In this embodiment, the candidate probes and the decoder probes should be of sufficient length (and the decoding step run under suitable

conditions) to allow specificity; i.e. each candidate probe binds to its corresponding decoder probe with sufficient specificity to allow the distinction of each candidate probe.

5 In a preferred embodiment, the DBLs are either directly or indirectly labeled. By "labeled" herein is meant that a compound has at least one element, isotope or chemical compound attached to enable the detection of the compound. In general, labels fall into three classes: a) isotopic labels, which may be radioactive or heavy isotopes; b) magnetic, electrical, thermal; and c) colored or luminescent dyes; although labels include enzymes and particles such as magnetic particles as well. Preferred labels include luminescent labels, particularly fluorescent labels as outlined above for optical signatures. In a preferred embodiment, the DBL is directly labeled, that is, the DBL comprises a label. In an alternate 10 embodiment, the DBL is indirectly labeled; that is, a labeling binding ligand (LBL) that will bind to the DBL is used. In this embodiment, the labeling binding ligand-DBL pair can be as described above for IBL-DBL pairs.

Accordingly, the identification of the location of the individual beads (or subpopulations of beads) is done using one or more decoding steps comprising a binding between the labeled DBL and either the 15 IBL or the bioactive agent (i.e. a hybridization between the candidate probe and the decoder probe when the bioactive agent is a nucleic acid). After decoding, the DBLs can be removed and the array can be used; however, in some circumstances, for example when the DBL binds to an IBL and not to the bioactive agent, the removal of the DBL is not required (although it may be desirable in some circumstances). In addition, as outlined herein, decoding may be done either before the array is used 20 in an assay, during the assay, or after the assay.

In one embodiment, a single decoding step is done. In this embodiment, each DBL is labeled with a unique label, such that the the number of unique tags is equal to or greater than the number of bioactive agents (although in some cases, "reuse" of the unique labels can be done, as described herein; similarly, minor variants of candidate probes can share the same decoder, if the variants are 25 encoded in another dimension, i.e. in the bead size or label). For each bioactive agent or IBL, a DBL is made that will specifically bind to it and contains a unique tag, for example one or more fluorochromes. Thus, the identity of each DBL, both its composition (i.e. its sequence when it is a nucleic acid) and its label, is known. Then, by adding the DBLs to the array containing the bioactive agents under conditions which allow the formation of complexes (termed hybridization complexes 30 when the components are nucleic acids) between the DBLs and either the bioactive agents or the IBLs, the location of each DBL can be elucidated. This allows the identification of the location of each bioactive agent; the random array has been decoded. The DBLs can then be removed, if necessary, and the target sample applied.

In a preferred embodiment, the number of unique labels is less than the number of unique bioactive 35 agents, and thus a sequential series of decoding steps are used. To facilitate the discussion, this

embodiment is explained for nucleic acids, although other types of bioactive agents and DBLs are useful as well. In this embodiment, decoder probes are divided into  $n$  sets for decoding. The number of sets corresponds to the number of unique tags. Each decoder probe is labeled in  $n$  separate reactions with  $n$  distinct tags. All the decoder probes share the same  $n$  tags. The decoder probes are  
5 pooled so that each pool contains only one of the  $n$  tag versions of each decoder, and no two decoder probes have the same sequence of tags across all the pools. The number of pools required for this to be true is determined by the number of decoder probes and the  $n$ . Hybridization of each pool to the array generates a signal at every address. The sequential hybridization of each pool in turn will generate a unique, sequence-specific code for each candidate probe. This identifies the candidate  
10 probe at each address in the array. For example, if four tags are used, then  $4 \times n$  sequential hybridizations can ideally distinguish  $4^n$  sequences, although in some cases more steps may be required. After the hybridization of each pool, the hybrids are denatured and the decoder probes removed, so that the probes are rendered single-stranded for the next hybridization (although it is also possible to hybridize limiting amounts of target so that the available probe is not saturated. Sequential  
15 hybridizations can be carried out and analyzed by subtracting pre-existing signal from the previous hybridization).

An example is illustrative. Assuming an array of 16 probe nucleic acids (numbers 1-16), and four unique tags (four different fluors, for example; labels A-D). Decoder probes 1-16 are made that correspond to the probes on the beads. The first step is to label decoder probes 1-4 with tag A, decoder probes 5-8 with tag B, decoder probes 9-12 with tag C, and decoder probes 13-16 with tag D.  
20 The probes are mixed and the pool is contacted with the array containing the beads with the attached candidate probes. The location of each tag (and thus each decoder and candidate probe pair) is then determined. The first set of decoder probes is then removed. A second set is added, but this time, decoder probes 1, 5, 9 and 13 are labeled with tag A, decoder probes 2, 6, 10 and 14 are labeled with  
25 tag B, decoder probes 3, 7, 11 and 15 are labeled with tag C, and decoder probes 4, 8, 12 and 16 are labeled with tag D. Thus, those beads that contained tag A in both decoding steps contain candidate probe 1; tag A in the first decoding step and tag B in the second decoding step contain candidate probe 2; tag A in the first decoding step and tag C in the second step contain candidate probe 3; etc.

In one embodiment, the decoder probes are labeled in situ; that is, they need not be labeled prior to the decoding reaction. In this embodiment, the incoming decoder probe is shorter than the candidate probe, creating a 5' "overhang" on the decoding probe. The addition of labeled ddNTPs (each labeled with a unique tag) and a polymerase will allow the addition of the tags in a sequence specific manner, thus creating a sequence-specific pattern of signals. Similarly, other modifications can be done, including ligation, etc.  
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In addition, since the size of the array will be set by the number of unique decoding binding ligands, it is possible to "reuse" a set of unique DBLs to allow for a greater number of test sites. This may be  
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done in several ways; for example, by using some subpopulations that comprise optical signatures. Similarly, the use of a positional coding scheme within an array; different sub-bundles may reuse the set of DBLs. Similarly, one embodiment utilizes bead size as a coding modality, thus allowing the reuse of the set of unique DBLs for each bead size. Alternatively, sequential partial loading of arrays with beads can also allow the reuse of DBLs. Furthermore, "code sharing" can occur as well.

In a preferred embodiment, the DBLs may be reused by having some subpopulations of beads comprise optical signatures. Thus, agents 1-100 are on non-labeled beads; agents 101-200 are on beads comprising a first label, etc. The unique combinations generated in this scheme allow the reuse of the DBL labels.

In a preferred embodiment, a spatial or positional coding system is done. In this embodiment, there are sub-bundles or subarrays (i.e. portions of the total array) that are utilized. By analogy with the telephone system, each subarray is an "area code", that can have the same tags (i.e. telephone numbers) of other subarrays, that are separated by virtue of the location of the subarray. Thus, for example, the same unique tags can be reused from bundle to bundle. Thus, the use of 50 unique tags in combination with 100 different subarrays can form an array of 5000 different bioactive agents. In this embodiment, it becomes important to be able to identify one bundle from another; in general, this is done either manually or through the use of marker beads, i.e. beads containing unique tags for each subarray.

In alternative embodiments, additional encoding parameters can be added, such as microsphere size. For example, the use of different size beads may also allow the reuse of sets of DBLs; that is, it is possible to use microspheres of different sizes to expand the encoding dimensions of the microspheres. Optical fiber arrays can be fabricated containing pixels with different fiber diameters or cross-sections; alternatively, two or more fiber optic bundles, each with different cross-sections of the individual fibers, can be added together to form a larger bundle; or, fiber optic bundles with fiber of the same size cross-sections can be used, but just with different sized beads. With different diameters, the largest wells can be filled with the largest microspheres and then moving onto progressively smaller microspheres in the smaller wells until all size wells are then filled. In this manner, the same dye ratio could be used to encode microspheres of different sizes thereby expanding the number of different oligonucleotide sequences or chemical functionalities present in the array. Although outlined for fiber optic substrates, this as well as the other methods outlined herein can be used with other substrates and with other attachment modalities as well.

In a preferred embodiment, the coding and decoding is accomplished by sequential loading of the microspheres into the array. As outlined above for spatial coding, in this embodiment, the optical signatures can be "reused". In this embodiment, the library of microspheres each comprising a different bioactive agent (or the subpopulations each comprise a different bioactive agent), is divided

into a plurality of sublibraries; for example, depending on the size of the desired array and the number of unique tags, 10 sublibraries each comprising roughly 10% of the total library may be made, with each sublibrary comprising roughly the same unique tags. Then, the first sublibrary is added to the fiber optic bundle comprising the wells, and the location of each bioactive agent is determined, generally through the use of DBLs. The second sublibrary is then added, and the location of each bioactive agent is again determined. The signal in this case will comprise the signal from the "first" DBL and the "second" DBL; by comparing the two matrices the location of each bead in each sublibrary can be determined. Similarly, adding the third, fourth, etc. sublibraries sequentially will allow the array to be filled.

In a preferred embodiment, codes can be "shared" in several ways. In a first embodiment, a single code (i.e. IBL/DBL pair) can be assigned to two or more agents if the target analytes differ sufficiently in their binding strengths. For example, two nucleic acid probes used in an mRNA quantitation assay can share the same code if the ranges of their hybridization signal intensities do not overlap. This can occur, for example, when one of the target sequences is always present at a much higher concentration than the other. Alternatively, the two target sequences might always be present at a similar concentration, but differ in hybridization efficiency.

Alternatively, a single code can be assigned to multiple agents if the agents are functionally equivalent. For example, if a set of oligonucleotide probes are designed with the common purpose of detecting the presence of a particular gene, then the probes are functionally equivalent, even though they may differ in sequence. Similarly, if classes of analytes are desired, all probes for different members of a class such as kinases or G-protein coupled receptors could share a code. Similarly, an array of this type could be used to detect homologs of known genes. In this embodiment, each gene is represented by a heterologous set of probes, hybridizing to different regions of the gene (and therefore differing in sequence). The set of probes share a common code. If a homolog is present, it might hybridize to some but not all of the probes. The level of homology might be indicated by the fraction of probes hybridizing, as well as the average hybridization intensity. Similarly, multiple antibodies to the same protein could all share the same code.

It should be noted that while the decoding step is discussed prior to the assay step, decoding need not be done prior to the assay; decoding may occur before, during or after the assay is performed.

Once made, the compositions of the invention find use in a number of applications. In general, there are two major applications: a) high throughput assays of candidate agents to detect interactions with target analytes, and b) high throughput assays for the detection of the presence or absence of target analytes, for example nucleic acids and proteins.



In a preferred embodiment, assay methods are run for the detection of the interaction (binding) of candidate agents with target analytes. That is, there are a wide variety of assays which are traditionally run to detect novel interactions; for example, chemical libraries are screened to evaluate their binding and/or bioeffect on any number of target analytes. Accordingly, the methods provide for the detection of binding of candidate agents to target analytes. By "binding" or "interaction" herein is meant a permanent or transitory physical interaction of the candidate agent and the target analyte.

By "target analyte" or "analyte" or grammatical equivalents herein is meant any atom, molecule, ion, molecular ion, compound or particle to be either detected or evaluated for binding partners. As will be appreciated by those in the art, a large number of analytes may be used in the present invention; basically, any target analyte can be used which binds a bioactive agent or for which a binding partner (i.e. drug candidate) is sought.

Suitable analytes include organic and inorganic molecules, including biomolecules. When detection of a target analyte is done, suitable target analytes include, but are not limited to, an environmental pollutant (including pesticides, insecticides, toxins, etc.); a chemical (including solvents, polymers, organic materials, etc.); therapeutic molecules (including therapeutic and abused drugs, antibiotics, etc.); biomolecules (including hormones, cytokines, proteins including enzymes, nucleic acids, lipids, carbohydrates, cellular membrane antigens and receptors (neural, hormonal, nutrient, and cell surface receptors) or their ligands, etc); whole cells (including procaryotic (such as pathogenic bacteria) and eukaryotic cells, including mammalian tumor cells); viruses (including retroviruses, herpesviruses, adenoviruses, lentiviruses, etc.); and spores; etc. Particularly preferred analytes are nucleic acids and proteins.

In a preferred embodiment, the target analyte is a protein. As will be appreciated by those in the art, there are a large number of possible proteinaceous target analytes that may be detected or evaluated for binding partners using the present invention. Suitable protein target analytes include, but are not limited to, (1) immunoglobulins; (2) enzymes (and other proteins); (3) hormones and cytokines (many of which serve as ligands for cellular receptors); and (4) other proteins.

In a preferred embodiment, the target analyte is a nucleic acid. These assays find use in a wide variety of applications, as is more fully outlined below.

In general, assays that are used to probe for novel interactions can be run in a variety of ways, as will be appreciated by those in the art. In general, since the assays are run within beads, assays requiring wash steps are not preferred. Accordingly, there are four major ways these assays may be accomplished: a) the candidate agent comprises a detectable moiety such as a fluorochrome that changes its detectable properties upon binding to the target analyte; b) the target analyte comprises the detectable moiety that alters upon binding to the candidate agent; c) both the target analyte and

the candidate agent comprise detectable moieties that alter their properties upon binding; or d) one of the assay components comprises a detectable moiety that alters upon the binding of the agent to the target.

5 In a preferred embodiment, the candidate agent comprises a detectable moiety that changes its properties or characteristics upon binding to the target analyte. For example, as is well known in the art, many fluorochromes alter their fluorescent properties as a result of the relative hydrophobicity of their environment. Thus, for example, some fluorochromes frequently exhibit low fluorescence in aqueous environments, such as free in aqueous solution. However, upon sequestration into a hydrophobic environment, for example a binding pocket of a target analyte, the fluor increases its  
10 signal.

In a preferred embodiment, the target analyte comprises a detectable moiety that changes its properties or characteristics upon binding to the candidate agent. Thus, for example, as outlined above, the binding pocket of an enzyme may comprise a solvent accessible fluor that alters its fluorescent properties upon binding of a candidate agent.

15 In a preferred embodiment, both the target and the candidate agent comprise detectable moieties that alter their properties upon binding. For example, fluorescence resonance energy transfer (FRET) systems are well known. In a preferred embodiment, the system comprises detectable molecules formed of two fluorescent proteins, i.e., blue and green fluorescent protein (BFP and GFP), and other similar molecules. As is known in the art, constructs of GFP and BFP that hold these two proteins in  
20 close proximity allow fluorescence resonance energy transfer (FRET). That is, the excitation spectra of the GFP overlaps the emission spectra of the BFP. Accordingly, exciting the BFP results in GFP emission. If the candidate agent and target each comprise one of these labels, the interaction of these two form a detectable "FRET complex"; if the two do not interact, there is no GFP emission at the BFP excitation wavelength. The system is further described in Xu et al., Nucleic Acid Res.  
25 26(8):2034 (1998); and Miyawaki et al., Nature 388(6645):882-887 (1997), both of which are incorporated by reference.

In a preferred embodiment, it is a component of the assay system that comprises a detectable moiety that alters as a result of the interaction of the target and candidate agent. For example, intercalators are molecules, many of which are fluorescent, that preferentially insert into double stranded nucleic  
30 acid. Upon binding, generally as a result of moving from a hydrophilic to a relatively hydrophobic environment, the fluorescence of the intercalator increases.

Similarly, in a preferred embodiment, the assay is directed to screening for enzymatic inhibitors, and the assay includes a substrate that becomes detectable as the enzyme acts upon it. That is, there are a wide variety of chromogenic and fluorogenic enzymatic substrates known that can be added to the

assay system. If the candidate agent is an effective inhibitor, a loss of signal is seen. Suitable enzymes for screening for inhibitors include, but are not limited to, viral, bacterial and parasitic enzymes and therapeutically relevant enzymes including, but not limited to, the cathepsins, caspases, other proteases, etc.

- 5 In a preferred embodiment, the compositions are used to probe a sample solution for the presence or absence of a target analyte, including the quantification of the amount of target analyte present. Thus, for example, a preferred embodiment utilizes nucleic acid probes as the candidate agents, to screen samples for the presence or absence of certain target sequences.

10 Thus, in a preferred embodiment, the probes are used in genetic diagnosis. For example, probes can be made using the techniques disclosed herein to detect target sequences such as the gene for nonpolyposis colon cancer, the BRCA1 breast cancer gene, P53, which is a gene associated with a variety of cancers, the Apo E4 gene that indicates a greater risk of Alzheimer's disease, allowing for easy presymptomatic screening of patients, mutations in the cystic fibrosis gene, cytochrome p450s or any of the others well known in the art.

- 15 In an additional embodiment, viral and bacterial detection is done using the complexes of the invention. In this embodiment, probes are designed to detect target sequences from a variety of bacteria and viruses. For example, current blood-screening techniques rely on the detection of anti-HIV antibodies. The methods disclosed herein allow for direct screening of clinical samples to detect HIV nucleic acid sequences, particularly highly conserved HIV sequences. In addition, this allows  
20 direct monitoring of circulating virus within a patient as an improved method of assessing the efficacy of anti-viral therapies. Similarly, viruses associated with leukemia, HTLV-I and HTLV-II, may be detected in this way. Bacterial infections such as tuberculosis, chlamydia and other sexually transmitted diseases, may also be detected.

25 In a preferred embodiment, the nucleic acids of the invention find use as probes for toxic bacteria in the screening of water and food samples. For example, samples may be treated to lyse the bacteria to release its nucleic acid, and then probes designed to recognize bacterial strains, including, but not limited to, such pathogenic strains as, Salmonella, Campylobacter, Vibrio cholerae, Leishmania, enterotoxigenic strains of E. coli, and Legionnaire's disease bacteria. Similarly, bioremediation strategies may be evaluated using the compositions of the invention.

- 30 In a further embodiment, the probes are used for forensic "DNA fingerprinting" to match crime-scene DNA against samples taken from victims and suspects.

In an additional embodiment, the probes in an array are used for sequencing by hybridization.

5 The present invention also finds use as a methodology for the detection of mutations or mismatches in target nucleic acid sequences. For example, recent focus has been on the analysis of the relationship between genetic variation and phenotype by making use of polymorphic DNA markers. Previous work utilized short tandem repeats (STRs) as polymorphic positional markers; however, recent focus is on the use of single nucleotide polymorphisms (SNPs), which occur at an average frequency of more than 1 per kilobase in human genomic DNA. Some SNPs, particularly those in and around coding sequences, are likely to be the direct cause of therapeutically relevant phenotypic variants. There are a number of well known polymorphisms that cause clinically important phenotypes; for example, the apoE2/3/4 variants are associated with different relative risk of Alzheimer's and other diseases (see 10 Cordor et al., Science 261(1993). Multiplex PCR amplification of SNP loci with subsequent hybridization to oligonucleotide arrays has been shown to be an accurate and reliable method of simultaneously genotyping at least hundreds of SNPs; see Wang et al., Science, 280:1077 (1998); see also Schafer et al., Nature Biotechnology 16:33-39 (1998). The compositions of the present invention may easily be substituted for the arrays of the prior art.

15 In a preferred embodiment, two-color competitive hybridization assays are run. These assays can be based on traditional sandwich assays. The beads contain a capture sequence located on one side (upstream or downstream) of the SNP, to capture the target sequence. Two SNP allele-specific probes, each labeled with a different fluorophor, are hybridized to the target sequence. The genotype can be obtained from a ratio of the two signals, with the correct sequence generally exhibiting better binding. This has an advantage in that the target sequence itself need not be labeled. In addition, 20 since the probes are competing, this means that the conditions for binding need not be optimized. Under conditions where a mismatched probe would be stably bound, a matched probe can still displace it. Therefore the competitive assay can provide better discrimination under those conditions. Because many assays are carried out in parallel, conditions cannot be optimized for every probe simultaneously. Therefore, a competitive assay system can be used to help compensate for non- 25 optimal conditions for mismatch discrimination.

In a preferred embodiment, dideoxynucleotide chain-termination sequencing is done using the compositions of the invention. In this embodiment, a DNA polymerase is used to extend a primer using fluorescently labeled ddNTPs. The 3' end of the primer is located adjacent to the SNP site. In 30 this way, the single base extension is complementary to the sequence at the SNP site. By using four different fluorophors, one for each base, the sequence of the SNP can be deduced by comparing the four base-specific signals. This may be done in several ways. In a first embodiment, the capture probe can be extended; in this approach, the probe must either be synthesized 5'-3' on the bead, or attached at the 5' end, to provide a free 3' end for polymerase extension. Alternatively, a sandwich type assay can be used; in this embodiment, the target is captured on the bead by a probe, then a 35 primer is annealed and extended. Again, in the latter case, the target sequence need not be labeled.

In addition, since sandwich assays require two specific interactions, this provides increased stringency which is particularly helpful for the analysis of complex samples.

In addition, when the target analyte and the DBL both bind to the agent, it is also possible to do detection of non-labelled target analytes via competition of decoding.

5 In a preferred embodiment, the compositions of the invention are used to screen bioactive agents to find an agent that will bind, and preferably modify the function of, a target molecule. As above, a wide variety of different assay formats may be run, as will be appreciated by those in the art.

10 In a preferred embodiment, the binding of the bioactive agent and the target analyte is specific; that is, the bioactive agent specifically binds to the target analyte. By "specifically bind" herein is meant that the agent binds the analyte, with specificity sufficient to differentiate between the analyte and other components or contaminants of the test sample. However, as will be appreciated by those in the art, it will be possible to detect analytes using binding which is not highly specific; for example, the systems may use different binding ligands, for example an array of different ligands, and detection of any particular analyte is via its "signature" of binding to a panel of binding ligands, similar to the manner in  
15 which "electronic noses" work. This finds particular utility in the detection of chemical analytes. The binding should be sufficient to remain bound under the conditions of the assay, including wash steps to remove non-specific binding, although in some embodiments, wash steps are not desired; i.e. for detecting low affinity binding partners. In some embodiments, for example in the detection of certain biomolecules, the dissociation constants of the analyte to the binding ligand will be less than about  
20  $10^{-4}$ - $10^{-6}$  M<sup>-1</sup>, with less than about  $10^{-5}$  to  $10^{-9}$  M<sup>-1</sup> being preferred and less than about  $10^{-7}$ - $10^{-9}$  M<sup>-1</sup> being particularly preferred.

Generally, a sample containing a target analyte (whether for detection of the target analyte or screening for binding partners of the target analyte) is added to the array, under conditions suitable for binding of the target analyte to at least one of the bioactive agents, i.e. generally physiological  
25 conditions. The sample or solution can additionally comprise a variety of other reagents. These include reagents like buffers, co-factors, enzyme substrates, intercalators, salts, neutral proteins, e.g. albumin, detergents, etc which may be used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Also reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used.

30 Either before, during or after the introduction of the sample to the microspheres the scissile linkages may be cleaved, releasing the candidate agents into the bead.

An interaction between the candidate agent and the target analyte is then detected. As outlined herein, this can be done in a variety of ways, depending on whether screening assays or detection

assays are done, although both types of assays rely on changes in detectable signals, generally optical signals, as a result of the interaction of the candidate agent and the target analyte.

When the assay is directed to the detection of the presence or absence of a target analyte, generally through the use of a change in an optical signal. This change can occur via many different mechanisms. A few examples include the binding of a dye-tagged analyte to the bead, the production of a dye species on or near the beads, the destruction of an existing dye species, a change in the optical signature upon analyte interaction with dye on bead, or any other optical interrogatable event.

In a preferred embodiment, the change in optical signal occurs as a result of the binding of a target analyte that is labeled, either directly or indirectly, with a detectable label, preferably an optical label such as a fluorochrome. Thus, for example, when a proteinaceous target analyte is used, it may be either directly labeled with a fluor, or indirectly, for example through the use of a labeled antibody. Similarly, nucleic acids are easily labeled with fluorochromes, for example during PCR amplification as is known in the art. Alternatively, upon binding of the target sequences, a hybridization indicator may be used as the label. Hybridization indicators preferentially associate with double stranded nucleic acid, usually reversibly. Hybridization indicators include intercalators and minor and/or major groove binding moieties. In a preferred embodiment, intercalators may be used; since intercalation generally only occurs in the presence of double stranded nucleic acid, only in the presence of target hybridization will the label light up. Thus, upon binding of the target analyte to a bioactive agent, there is a new optical signal generated at that site, which then may be detected.

Alternatively, in some cases, as discussed above, the target analyte such as an enzyme generates a species that is either directly or indirectly optical detectable.

Furthermore, in some embodiments, a change in the optical signature may be the basis of the optical signal. For example, the interaction of some chemical target analytes with some fluorescent dyes on the beads may alter the optical signature, thus generating a different optical signal.

As will be appreciated by those in the art, in some embodiments, the presence or absence of the target analyte may be done using changes in other optical or non-optical signals, including, but not limited to, surface enhanced Raman spectroscopy, surface plasmon resonance, radioactivity, etc.

When screening assays are done, detection proceeds via a change in a detectable signal as outlined above, generally using at least one of the four outlined mechanisms. The detectable signal is usually an optical signal, and preferably a fluorescent signal.

All references cited herein are incorporated by reference in their entirety.

## CLAIMS

We claim:

1. An array composition comprising:
  - a) a substrate with a surface comprising discrete sites; and
  - b) a population of microspheres comprising at least a first and a second subpopulation, wherein each subpopulation comprises:
    - i) a candidate bioactive agent linked to said microspheres via a scissile linkage; and
    - ii) an identifier moiety;wherein said microspheres are distributed on said surface.
2. An array composition according to claim 1 wherein said identifier moiety is an identifier binding ligand that will bind a decoder binding ligand such that the identification of the bioactive agent can be elucidated.
3. An array composition according to claim 2 wherein said identifier binding ligand is a nucleic acid probe.
4. An array composition according to claim 2 wherein said identifier binding ligand is a protein.
5. An array composition according to claim 1 wherein said identifier moiety is an optical signature.
6. An array composition according to claim 5 wherein said optical signature comprises a fluorescent dye.
7. An array composition according to claim 6 wherein said optical signature comprises two or more fluorescent dyes.
8. An array composition according to claim 1 wherein at least two subpopulations have different amounts of the same candidate agent.
9. An array composition comprising:
  - a) a substrate with a surface comprising discrete sites; and
  - b) a population of microspheres comprising at least a first and a second subpopulation, wherein said first and said second subpopulation each comprise the same candidate bioactive agent linked to said microspheres via a scissile linkage, wherein the amount of candidate agent on said first subpopulation is different from the amount of candidate agent on said second subpopulation;

wherein said microspheres are distributed on said surface.

10. An array composition according to claim 1 or 9 wherein said scissile linkage is a photocleavable linkage.

5 11. An array composition according to claim 1 or 9 wherein said scissile linkage is an enzyme cleavable linkage.

12. An assay method for detecting the binding of a candidate bioactive agent to a target analyte comprising:

10 a) adding a solution comprising at least one target analyte to a plurality of microspheres comprising at least a first and a second subpopulation, wherein each subpopulation comprises:

i) a candidate bioactive agent linked to said microspheres via a scissile linkage; and

ii) an identifier moiety;

b) cleaving said scissile linkage;

15 c) detecting the binding of at least one candidate bioactive agent to said target analyte.

13. A method according to claim 12 wherein said candidate bioactive agent comprises a fluorochrome that exhibits a change in fluorescence as a result of binding to said target analyte.

14. A method according to claim 12 wherein said target analyte comprises a fluorochrome that exhibits a change in fluorescence as a result of binding to said candidate agent.

20 15. A method according to claim 12 wherein said solution comprises a fluorochrome that exhibits a change in fluorescence as a result of the binding of said candidate agent to said target analyte.

16. A method according to claim 12 wherein said scissile linkage is a photocleavable linkage.

17. A method according to claim 12 wherein said scissile linkage is an enzymatically cleavable linkage.

25 18. A method according to claim 12 wherein said beads are distributed on a surface at discrete sites.

19. A method according to claim 13 wherein said identifier moiety is an identifier binding ligand that will bind a decoder binding ligand such that the identification of the bioactive agent can be elucidated.

20. A method according to claim 19 wherein said identifier binding ligand is a nucleic acid probe.



21. A method according to claim 19 wherein said identifier binding ligand is a protein.
22. A method according to claim 19 wherein said identifier moiety is an optical signature.
23. A method according to claim 22 wherein said optical signature comprises a fluorescent dye.
24. A method according to claim 22 wherein said optical signature comprises two or more fluorescent dyes.
- 5
25. A method according to claim 12 wherein at least two subpopulations have different amounts of the same candidate agent.
26. A method according to claim 12 wherein said target analyte is a protein.
27. A method according to claim 26 wherein said protein is an enzyme.

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/03370

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N33/543

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N B01J C07K C07B C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	WO 98 40726 A (TUFTS COLLEGE ;MICHAEL KARRI C (US); WALT DAVID R (US)) 17 September 1998 (1998-09-17) the whole document ---	1-7, 9-24,26, 27
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Further documents are listed in the continuation of box C.



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Date of the actual completion of the international search

10 July 2000

Date of mailing of the international search report

17/07/2000

Name and mailing address of the ISA

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>7</sup> : <b>G01N 21/64, 21/77</b>		A3	(11) International Publication Number: <b>WO 00/13004</b>
			(43) International Publication Date: 9 March 2000 (09.03.00)
(21) International Application Number: PCT/US99/19624 (22) International Filing Date: 26 August 1999 (26.08.99) (30) Priority Data: 09/140,352          26 August 1998 (26.08.98)          US (71) Applicant (for all designated States except US): TRUSTEES OF TUFTS COLLEGE [US/US]; 136 Harrison Avenue, Boston, MA 02111 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): WALT, David, R. [US/US]; 4 Candlewick Close, Lexington, MA 02178 (US). DICKINSON, Todd, A. [US/US]; Apartment 1133, 3435 Lebon Drive, San Diego, CA 92122 (US). (74) Agents: BREZNER, David, J. et al.; Flehr Hobbach Test Albritton & Herbert LLP, Suite 3400, 4 Embarcadero Center, San Francisco, CA 94111-4187 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  Published With international search report. With amended claims.  (88) Date of publication of the international search report: 8 June 2000 (08.06.00)  Date of publication of the amended claims: 17 August 2000 (17.08.00)	
(54) Title: COMBINATORIAL POLYMER SYNTHESIS OF SENSORS FOR POLYMER-BASED SENSOR ARRAYS			
(57) Abstract			
<p>A combinatorial synthesis method for fabricating unique families of discrete copolymer sensors and copolymer gradient sensors is provided. The method employs combinatorial copolymer synthesis of discrete monomer or oligomer combinations as well as spatially-varying combinations for generating large numbers of analyte-discriminating sensors from a limited selection of initial monomer and oligomer compositions. The method can be applied to either analyte-specific sensors or sensor arrays or semi-selective sensors and cross-reactive sensor arrays which employ virtually any known physicochemical transduction mechanism for detecting analytes. Since the analyte response characteristics of such copolymer sensors are not limited to a linear proportional ratio of the monomer or oligomer combinations employed, the resulting copolymer sensors provide for increased diversity in sensor and sensor array response characteristics for discriminating between a variety of materials and for detecting and identifying analytes in fluid samples.</p>			

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CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

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## COMBINATORIAL POLYMER SYNTHESIS OF SENSORS FOR POLYMER-BASED SENSOR ARRAYS

### FIELD OF THE INVENTION

This invention relates to a method for combinatorial polymer synthesis of sensors for polymer-based  
5 sensor arrays and, more particularly, to copolymer sensor compositions, their synthesis and application  
in chemical sensor arrays.

### BACKGROUND OF THE INVENTION

Combinatorial synthesis methods are known in the art and have been successfully employed in a  
growing number of research areas including drug discovery [E.M. Gordon, et al., *Acc. Chem. Res.*  
10 29:144 (1996); M.J. Plunkett, et al., *Scientific American*, April 1997, p. 69], drug optimization [Hobbs, et  
al., *Acc. Chem. Res.* 29:114 (1996)], complex sequence-selective receptor molecule synthesis [W.C.  
Still, *Acc. Chem. Res.* 29:155 (1996)], catalytic antibody production [P.G. Schultz, et al., *Science*  
269:1835 (1995)], inorganic superconductor synthesis [X.D. Xiang, et al., *Science* 268:1738 (1995)],  
inorganic magnetoresistance materials synthesis [G. Briceno, et al., *Science* 270:273 (1995)] and  
15 inorganic luminescent materials synthesis [E. Danielson, et al., *Nature* 389:944 (1997)].

Photochemical approaches to combinatorial synthesis have been disclosed in a number of references  
[see U.S. Patent Nos. 5,288,514 and 5,545,568 to Ellman; S.P.A. Fodor, et al., *Science* 251:767 (1991);  
U.S. Patent No. 5,424,186 and PCT Publication No. WO 92/10092 to Fodor, et al; U.S. Patent No.  
5,527,681 to Holmes, et al; J.W. Jacobs, et al., *TIB TECH* 12:19 (1994); U.S. Patent No. 5,412,087 to  
20 McGall, et al; and U.S. Patent No. 5,143,854 to Pirrung, et al;]. In these methods, photochemical  
syntheses of large arrays of biologically active compounds which are immobilized on solid substrates  
are disclosed using conventional photolithography methods. With these methods, immobilized polymer  
compounds are assembled in step-wise fashion using spatially localized photolabile masking groups  
in combination with conventional masks for selective illumination, blocking, cleavage, coupling and  
25 photochemical reaction of functional groups with immobilized polymer backbones. These references  
disclose methods for fabricating arrays of diverse biological compounds for chemical assays by way of



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a sequential build-up of molecular fragments onto a polymer backbone in a series of masking steps and photoinitiated reactions.

Lundstrom, et al., [I. Lundstrom, et al., *Nature* 352:47 (4 July 1991); I. Lundstrom, *Sensors and Actuators*, A56:75 (1996)] have disclosed a quasi-combinatorial method for fabricating chemical sensors  
5 in which simple sets of binary masks are used to produce a patterned, field-effect transistor (FET) array surface by sequential masking and evaporative deposition of layers of different metals on a semiconductor substrate. The metals are subsequently heated to combine the vapor deposited materials by either reaction or alloying to form a matrix of different compounds. This method provides for production of thin-film chemical-FET (ChemFET) sensor arrays with a variety of different film  
10 compositions deposited in distinct areas of the array.

Xiang, et al., [X.D. Xiang, et al., *Science* 268:1738 (1995)] have disclosed a quasicombinatorial method for fabricating superconducting thin films using a similar approach to that of Lundstrom, et al. The disclosed method relies on a combination of conventional thin film deposition methods and physical masking by reacting layers of deposited materials to generate a spatially defined library of solid-state  
15 thin film compositions of electronic, magnetic, or optical materials by parallel synthesis. Danielson, et al., [E. Danielson, et al., *Nature* 389:944 (1997)] have disclosed a quasi-combinatorial approach similar to that of Lundstrom and Xiang for rapidly synthesizing and prescreening candidate luminescent materials whose properties cannot be predicted by theoretical models. This method also relies on conventional physical masking and deposition of multiple thin film layers for production of a variety of  
20 layered combinations for subsequent reaction and synthesis via thermal oxidative annealing of the as-deposited, multi-layered, solid-state structures to form libraries of candidate compounds for evaluation.

Schultz [PCT Application No. PCT/US95/13278 (Ins. Publication No. WO 06/11878)] discloses a method for parallel deposition, synthesis and screening of arrays of diverse compounds at predetermined  
25 locations on a solid substrate. The method relies on delivering a plurality of components in any stoichiometry, or stoichiometric gradients, to predefined regions on a substrate and simultaneously reacting the components to form a plurality of different compounds. Disclosed methods for delivery of components include thin-film deposition techniques in combination with masking and photolithography, and dispensing methods where droplets or powders are delivered by a suitable dispenser. Disclosed  
30 methods for reacting the components include solution-based synthesis, template directed synthesis, photoinitiated reactions, polymerization, heating, annealing and crystallization. The disclosed method is used for rapid parallel synthesis and screening to identify optimized compositions through measurement and comparison of their known targeted properties.

With the above disclosed methods, various permutations and combinations of candidate materials,  
35 compounds, chemical functionality or elements are experimentally synthesized and screened to identify

optimum compositions or properties for a known application. With these methods, combinatorial synthesis techniques are typically used to efficiently synthesize, test and identify materials in prescreening a large number of organic or inorganic compounds for an optimum targeted reactivity or performance characteristic where the performance of candidate materials cannot be predicted by theory. With these methods, compositions which do not demonstrate optimum performance or preferred properties are typically eliminated and discarded as undesirable or impractical candidate materials for the targeted application.

In the field of chemical sensor technology, a variety of chemical sensor formats and transduction mechanisms have been developed for detection of analytes in gaseous and liquid samples. These sensors employ various sensing mechanisms, utilizing changes in chemical, physical, electrical, mechanical, optical, or thermal properties of sensor materials for detection and recognition of target analytes in contact [see *Chemical Sensors and Microinstrumentation*, ed. R.W. Murray, et al., American Chemical Soc. (Washington, D.C. 1989); R.W. Catrall, *Chemical Sensors*, Oxford Univ. Press, (New York 1997); *Handbook of Chemical and Biological Sensors*, ed. R.F. Taylor, et al., Inst. of Physics Publishing (Philadelphia 1996); G. Boisdé, et al., *Chemical and Biochemical Sensing with Optical Fibers and Waveguides*, Artech House (Boston 1996); and *Surface-Launched Acoustic Wave Sensors*, M. Thompson, et al., J. Wiley & Sons (New York 1997).

A number of electrical transduction mechanisms have been employed for chemical sensing of target analytes. Electrical sensors have been employed where the sensor transduction mechanism is due to changes in conductance, resistance, or interface potential. Conductivity sensors rely on adsorption of an analyte onto a sensor substrate interposed between two electrodes biased by an applied potential. With these sensors, the adsorbed analyte produces a change in charge carriers which creates a detectable change in current. Amperometric chemical sensors have been developed for measuring microamp currents produced by analyte adsorption between two sensor electrodes maintained at a fixed relative potential. In addition, amperometric sensors have been developed which rely on electrochemical reaction currents generated by redox reactions occurring at the sensor surface. Potentiometric sensors rely on interfacial electrochemical potentials created by binding or transport of charged analytes across thin membrane films which are designed with chemical binding or transport specificity to given analytes. In addition, potentiometric chemical sensors have been developed as ion-selective electrodes which utilize half-cell reaction voltages for electrochemical detection of specific analytes.

U.S. Patent No. 4,717,673 to Wrighton, et al., discloses electrode applications of conducting polymers where reversible interaction of a polymer with an analyte produces a characteristic voltage. U.S. Patent No. 5,696,314 to McCaffrey, et al., discloses an amperometric electrode where a characteristic current is produced by interaction of an analyte with an immobilized enzyme layer.

Additional electrical-based transduction mechanisms have been employed in sensors which rely on changes in resistance, inductance or capacitance due to analyte sorption. U.S. Patent Nos. 5,607,573 and 5,417,100 to Miller, et al, disclose applications of conductive and dielectric polymers in electrodes where analyte sorption produces detectable changes in conductivity of the polymers. U.S. Patent Nos. 5,571,401 and 5,698,089 to Lewis, et al., disclose sensors and sensor arrays that employ chemically sensitive resistors comprised of non-conducting polymers and conductive materials which produce a detectable change in resistance upon exposure to analytes. H.V.Shurmer, et al., *Sensors and Actuators B* 4:29 (1991) and P.N. Bartlett, et al., *Sensors and Actuators A* 23:911(1990) and *Sensors and Actuators A* 20:287(1989) disclose gas sensors which employ conducting polymers for detection of analyte vapors. U.S. Patent No. 5,417,100 to Miller, et al., discloses a conductive electrode vapor sensor comprised of a composite coating of a conductive polymer and a dielectric polymer which has an affinity for analyte vapors. U.S. Patent No. 5,312,762 to Guiseppi-Elie discloses a resistivity sensor which relies on a change in resistance of electroactive polymers when exposed to analytes.

More recently, electronic chemical sensors have been developed which rely on changes in solid-state electronic properties due to adsorption of analytes on polymer-coated field effect transistor (FET) or metal oxide field effect transistor (MOSFET) surfaces [see Y. Ito, et al., *Sensors and Actuators B* 1-3:348 (1983); Miyahara, et al., *Sensors and Actuators B* 7:1 (1985); Hanazato, et al., *IEEE Trans.*, vol. ED-33(1):47 (Jan. 1986); Caras, et al., *Anal. Chem.* 57(9):1920-1925 (1985); I. Lundstrom, et al., *Nature* 352:47 (4 July 1991); H.M. McConnell, et al., *Science* 257: 1906 (1992); I. Lundstrom, *Sensors and Actuators A* 56:75 (1996); U.S. Patent No. 5,466,348 to Holm-Kennedy]. U.S. Patent No. 4,909,921 to Ito discloses an electrochemical FET sensor for detecting chemical substances which employs a continuous hydrous polymer coating on an insulating layer and an enzyme-immobilized coating formed on a channel portion of the FET.

Mass-sensitive mechanical properties have also been employed as transduction mechanisms for detecting target analytes. Piezoelectric sensors, such as quartz crystal microbalances or surface acoustic wave devices, have been employed which rely on an oscillating crystal that produces a shift in oscillating frequency due to sorption of analytes on a sensor surface [see W.P. Carey, et al., *Anal. Chem.* 58:3077 (1986); K. Yokoyama, et al., *Anal.Chem.* 65:673 (1993); M.Rapp, et al., *Fresenius J.Anal. Chem.* 352:699 (1995)]. In typical applications of such piezoelectric sensors, carefully selected polymer coatings are applied to the surface for optimizing sensitivity and selectivity towards specific target analytes [see W.P. Carey, et al., *Anal. Chem.* 58:149 (1986); E.T. Zellers, et al., *Anal.Chem.* 67:1092 (1995); J.W. Grate; et al., *Anal.Chem.* 67:2162 (1995)]. Generally, such devices rely on selective interaction of an analyte with a thin film coating placed on an active surface of the sensor. By binding or partitioning of an analyte due to the presence of the surface coating, the resonant frequency of the sensor is reduced in proportion to the increased mass of analyte. By proper selection of sorptive coatings, these sensors are capable of detecting analytes by measurement of changes in crystal oscillating frequency.

U.S. Patent No. 4,596,697 to Ballato discloses a piezoelectric sensor array which employs a plurality of coated resonators embedded in a single piezoelectric crystal where reactive coatings are utilized for detection of target analytes. In U.S. Patent Nos. 5,719,324 to Thundat, et al., and 5,445,008 to Wachter, et al., micro-cantilever piezoelectric sensors are disclosed which rely on resonant frequency changes  
5 in a micro-cantilever oscillated by a piezoelectric transducer where such changes are produced by adsorption of analytes on a microcantilever coated with a sorptive material.

Thompson, et al., have recently reviewed the state of the art of surface acoustic wave sensor ("SAW") technology [see M. Thompson, *Surface-Launched Acoustic Wave Sensors*, J.Wiley & Son (New York 1997)]. These devices are much more sensitive to oscillation frequency changes than conventional  
10 quartz crystal microbalances due to the substantial increase in detectable frequency range available with SAW devices. In U.S. Patent Nos. 4,895,017 to Pyke, et al., 5,151,110 to Bein, et al., 5,235,235 to Martin, et al., and 5,25,704 to Mariani, et al., surface acoustic wave sensors are disclosed which rely on the change in resonant frequency of propagated surface waves due to adsorption of analytes by a sorptive surface coating. Surface acoustic wave sensor arrays which have employed a variety of  
15 chemically selective coatings have been disclosed in U.S. Patent No. 5,464,608 to Lokshin, et al., and by Rapp, et al., [M.Rapp, et al, *Fresenius J.Anal.Chem.* 352:699 (19995)]. Criteria for optimal selection of SAW polymer coatings have been discussed by W.P. Carey, et al., *Anal. Chem.* 58: 149 (1986), E.T. Zellers, et al, *Anal.Chem.* 67:1092 (1995), and J.W. Grate, et al., *Anal.Chem.* 67:2162 (1995).

Optical and electro-optical mechanisms have also been employed as transduction methods for sensors  
20 that rely on changes in optical response for detecting analytes [see G.Boisde and A.Hammer, *Chemical and Biochemical Sensing with Optical Fibers and Waveguides*, Artech House (Boston 1996)]. Thus, optical absorbance, emittance, transmittance, reflectance, luminescence, interference, polarization, or surface plasmon resonance may be monitored for detecting analytes. With these methods, the sample or sensor is typically illuminated with incident light energy having a certain optical or spectral  
25 characteristic and the sensor-analyte interaction produces an optically detectable response which is indicative of the analyte.

Optical sensors and sensing methods have been disclosed which rely on changes in refractive index due to sorption of analytes at a sensor surface Esee Kawahara. et al., *Anal. Chim. Acta* 151:315 (1983); Sutherland, et al., *Anal. Lett.* 17:43 (1984); Guiliani, et al., *Sens. Actuat.* 6:107 (1984); V.S.Y. Lin, et al.,  
30 *Science* 278:840(31 Oct. 1997)]. Optical sensors which employ interferometric measurements have been disclosed by Butler [*Appl.Phys.Lett.* 45:1107 (1984)] and Dessy [*Anal.Chem.* 57:1188A (1985)]. U.S. Patent No. 5,606,633 to Groger, et al., discloses a transduction method which relies on monitoring the intensity ratios of TM and TE polarization of an incident light beam where attenuation of TM polarization occurs due to adsorption of an analyte at the sensor surface.

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Fiber optic sensors which employ optical fibers, fiber optic arrays and light absorbing dyes are particularly useful as optical sensors and have been disclosed by a number of workers [see W.R. Seitz, *C.R.C. Crit. Rev. Anal. Chem.* 19:135 (1988); *Molecular Luminescence Spectroscopy, Methods and Applications*, ed. S.G. Schulman, J. Wiley & Sons (New York 1988); D.R. Walt, et al., in *Chemical Sensors and Microinstrumentation*, ACS Symposium 403, p. 252, American Chemical Soc. (Washington, D.C. 1989); S.M. Barnard, et al., *Environ. Sci. Technol.* 25(7):1301 (1991); S.M. Barnard, et al., *Nature* 353:338 (26 Sept 1991); O.S. Wolfbeis in *Fiber Optic Chemical Sensors*, vol. 2, CRC Press (Boca Raton, FL 1991)]. U.S. Patent Nos. 5,244,636 and 5,250,264 to Walt, et al., disclose methods for attaching a plurality of polymer-dye combinations, representing multiple chemical functionalities, as optical sensor elements in a fiber optic array where sensor elements are selected due to their sensitivity and selectivity for particular analytes. In order to provide for discrimination and detection of analytes of interest by these methods, sensor elements typically must be capable of producing a characteristic optical response in the presence of an analyte when subjected to excitation light energy.

Surface plasmon resonance methods have recently been disclosed as useful optical transduction methods for chemical sensors in detecting of analytes [see K. Matsubara, et al., *Applied Optics* 27:1160 (1988); D.C. Cullen, et al., *Sensors and Actuators B*:576 (1990); Villuendas, et al., *Sensors and Actuators A*21 :1142 (1990); I.Garcés, et al., *Sensors and Actuators B*7:771 (1992); J.Melendez, et al., *Sensors and Actuators B*39(1-3):375 (1997)]. U.S. Patent Nos. 5,255,075 to Cush, and 5,359,681 or 5,647,030 to Jorgenson, et al]. Cush discloses a transduction method which relies on an angular shift in the resonance angle of incident polarized light as a means for sensing analyte absorption on the sensor. U.S. Patent Nos. 5,359,681 and 5,647,030 to Jorgenson, et al., disclose use of thin film coatings as either dynamic range controlling layers, for modifying the range in indices of refraction of sensors, or reactive layers, for interacting with specific analytes to produce a detectable shift in an effective surface refractive index due to absorption of an analyte by the film layer.

While the above sensing methods typically rely on the sensitivity of a particular sensor to a specific analyte, more recently, chemical sensor array formats have been developed, in which a series of discrete, cross-reactive sensing regions are used in conjunction with pattern recognition schemes for detecting and identifying a broad range of analytes. With this cross-reactive sensor array approach, there is no need to employ sensor elements which are specifically sensitive to a particular analyte as the combined sensor responses within the sensor array are used for producing a characteristic array response to a diverse selection of analytes.

A variety of sensor transduction methods have been employed in such cross-reactive sensor arrays where sensor array elements may utilize any determinable physicochemical phenomenon which is characteristic of the sensor-analyte interaction and which provides a discriminating and detectable response. Thus, changes in electrical properties, such as conductance, resistance, interface potential, electrochemical half-cell voltage or reaction currents, optical properties, such as absorption, emission,

reflectance, transmittance, polarization, interference, or surface plasmon resonance, physical changes, such as swelling or contraction, mass changes due to surface sorption of analytes, or any other measurable physicochemical analyte-sensor interaction phenomena may be utilized for sensing analytes.

- 5 Such cross-sensing sensor array methods have been applied to surface acoustic wave sensor formats [J. Grate, et al., *Anal. Chem.* 63: 1719 (1991); S. Rose-Pehrsson, *Anal. Chem.* 60:2801 (1988); E. Zellers, et al., *Anal. Chem.* 67:1092 (1995)], electrochemical sensor formats [J. Stetter, et al., *Anal. Chem.* 58:860 (1986)], conductive polymer sensor formats [J. Hatfield, et al., *Sens. Actuators B* 18-19:221 (1994); M. Freund, et al., *Proc. Natl. Acad. Sci. U.S.A.* 92:2652 (1995); Lewis, et al., U.S. Patent Nos. 5,571,401 and 5,698,089], and piezoelectric sensor formats [W. Carey, et al., *Anal. Chem.* 60:2801 (1988); T. Thundat, et al., *Anal. Chem.* 67:519 (1995)].

- More recently, Walt, et al., have disclosed cross-reactive fiber optic array sensors which employ diverse polymer-dye combinations in conjunction with fiber optic bundles or arrays for detecting a variety of analytes [see J. White, et al., *Anal. Chem.* 68:2191 (1996) and T.A. Dickinson, et al., *Nature* 382:697 (1996)]. U.S. Patent No. 5,512,490 to Walt, et al., discloses a cross-reactive, fiber optic sensor array comprised of a plurality of polymer-dye combinations as sensor elements where the sensors are semi-selective for particular analytes. The disclosed array and detection method provides for detecting a variety of materials by applying pattern recognition techniques to the composite sensor array response to analytes.

- 20 Polymeric materials have particular utility as either sensor element matrices or surface coatings applied to sensor elements for enhancing sensor discriminating capabilities towards analytes. Thin polymer films are effective in modifying and enhancing sensor reactivity, response times, sensitivity, specificity and selectivity and may be successfully employed in most every sensor format. Due to the substantial flexibility and control of polymer structure and properties, polymer films may be chemically designed to preferentially adsorb or repel particular analytes, thereby significantly improving selectivity of a sensor. Polymer thin films may enhance chemical selectivity by either altering the partitioning of analytes or interferants between the sample medium and sensor or modifying the transport properties of analytes to the sensor surface.

- A variety of polymeric materials have been employed in cross-reactive sensor arrays as semi-selective sensor elements to provide for discrimination between target analytes. In these applications, analyte discrimination may be achieved by exploiting various polymer properties such as analyte solvation and partition coefficients [J.W. Grate, et al., *Anal. Chem.* 67:2162 (1995); J.W. Grate, et al., *Sens. Actuators B* 3:85 (1991)] molecular polarity [J. White, et al., *Anal. Chem.* 68:2191 (1996); T.A. Dickinson, et al., *Nature* 382:697 (1996)], dimensional changes or swelling upon exposure to analytes [J. White, et al., *Anal. Chem.* 68:2191 (1996); T.A. Dickinson, et al., *Nature* 382:697 (1996); M.C. Lonergan, et al., *Chem.*

*Mater.* 8:2298 (1996); S.M. Barnard, et al., *Environ. Sci. Technol.* 25:1301 (1991)], changes in conductivity upon exposure to an analyte [W.P. Carey, et al., *Anal. Chem.* 58:3077 (1986); S. Unde, et al., *Adv. Mater. Opt. Elect.* 6:151 (1996)], and analyte sorption characteristics [R.A. McGill, et al., *CHEMTECH* 9:27 (1994); J.W. Grate, et al., *Anal. Chem.* 68:913(1996)].

- 5 Irrespective of the transduction means employed for chemical sensors, a potentially significant limitation of current chemical sensors and sensor technology is the limited selection and choice of polymeric sensor materials and sensor coatings which can provide discriminating responses for detecting analytes of interest by producing a unique, determinable, characteristic sensor response to a variety of analytes. Thus, a new family of sensor materials which provides for expanding the number and diversity of
- 10 available sensor elements for use as discrete chemical sensors or in chemical sensor arrays is required for increasing the capabilities of chemical sensor arrays and improving the selectivity, sensitivity and detection of a variety of target analytes with such arrays.

#### SUMMARY OF THE INVENTION

- In general, the present invention provides for the development of families of unique chemical sensing
- 15 elements for use either as discrete sensors or as semi-selective sensor elements in cross-reactive chemical sensor arrays and a method for fabricating the same. The innovative method relies on combinatorial polymer synthesis of copolymer sensors from two or more distinct monomers, oligomers, or their derivatives, where the monomers or oligomers are polymerized in either discrete composition ratios or from continuously varying composition ratios. The synthesis of diverse polymers may likewise
- 20 be performed on beads as starting materials, leading to combinatorially functionalized beads. The method provides for the rapid development and generation of large families of innovative, semi-selective polymer sensor elements for use in chemical sensor arrays from a limited number of available monomer or oligomer starting materials. By increasing the number of unique sensor element types in a sensor array, an increasing amount of discriminating characteristic sensor response information is provided for
- 25 collection and analysis in detecting analytes of interest. By increasing the amount of discriminating information which is generated by such sensor arrays for processing by neural networks or other computer-based pattern recognition schemes, the detection capability and sensitivity of the sensor array to a variety of target analytes is greatly enhanced.

- The combinatorial polymer synthesis method for fabricating chemical sensors of the present invention
- 30 further provides for synthesizing a large family of copolymer sensors that produce more distinctive and characteristic responses to the presence of analytes of interest than would be anticipated when two or more different monomers or oligomers are proportionally combined in varying composition ratios and copolymerized to produce a new copolymer sensor matrix material. The response characteristics of such combinatorial polymer sensors are thus more unique and distinctive than the mere proportional

combination of starting monomer or oligomer materials would suggest. The ability to create such variations in the responses of sensor elements to target analytes, from copolymerization of two or more starting monomers or oligomers in varying proportions, represents a potentially powerful and useful approach for chemical sensor array development and fabrication for chemical sensing of an increasingly large number of target analytes.

The polymer sensor of the present invention can take on a variety of configurations for generating chemical sensor diversity through the combinatorial polymer synthesis method. In one embodiment, predetermined composition ratios of two or more distinct monomers or oligomers are combined and polymerized to form a unique copolymer matrix material useful as a polymer sensor element. In this embodiment, specific monomer or oligomer composition ratios produce discrete copolymer sensor elements which exhibit unique and characteristic responses for given target analytes. As more fully outlined below, this can be done in a variety of ways, including polymerizing the mixtures onto discrete sites of a substrate, or onto microspheres distributed onto the substrate, or by polymerizing the mixtures into beads which are distributed on the substrate. In an alternative embodiment, prepolymer mixtures of continuously varying ratios of two or more monomers or oligomers may be copolymerized to form a continuous copolymer gradient sensor which has a spatially varying polymer structure. In this embodiment, the continuous variation in composition ratio during polymerization of two or more distinct monomers or oligomers to form a copolymer sensor matrix creates a gradient sensor having a spatially varying copolymer structure with unique analyte response characteristics which vary according to spatial location along the copolymer structure gradient. With such gradient sensors, each location within the copolymer gradient provides a uniquely characteristic response to target analytes. With these embodiments, by utilizing predetermined varying composition ratios of a relatively small group of monomers or oligomers and combining them in predetermined permutations and combinations of prepolymer mixtures for copolymerization in differing proportions, an expansive set of unique combinatorial polymer sensors are made available in which each sensor exhibits a unique characteristic response to a variety of analytes.

The combinatorial polymer sensors and sensor arrays of the present invention may be employed with a variety of detection or transduction mechanisms for providing a characteristic and discriminating response to target analytes of interest. Sensors of the present invention have utility in a variety of sensing applications as either discrete sensors or in sensor arrays where, upon exposure of the copolymer sensors to analytes, interaction of analytes with the sensors produces a unique, characteristic, and determinable, time-varying, physicochemical response, said response detected by measuring a property selected from a group consisting of mass, temperature, heat, voltage, current, polarity, intensity, refractive index, polarization, phase, wavelength, frequency, periodicity, and dimension, said response being indicative of the presence or absence of certain analytes.



By way of example, optical sensors and sensor arrays of the present invention may employ characteristic spectral response signatures, such as variation in absorption, emission, transmission or reflection intensities or wavelengths, or, alternatively, spectral variations in interference, polarization, refractive index, periodicity, phase or frequency for identification and detection of analytes. Alternatively, 5 combinatorial polymer sensor coatings may be employed with piezoelectric sensors, such as quartz crystal microbalances, surface acoustic wave (SAW), or bulk acoustic wave (BAW) sensors and sensor arrays, where such coatings provide for discrimination and detection of analytes due to mass changes from sorption of analytes. In addition, analytes may be identified and distinguished by employing 10 combinatorial copolymer sensors and sensor arrays fabricated from dielectric and conducting polymers where sorption of target analytes produce unique and characteristic changes in resistance, conductance, capacitance, voltage or current. Other embodiments include combinatorial polymer sensors which rely on discriminating between temperature changes or heat generated due to adsorption of target analytes with micro-calorimeter sensors, ion-selective electrochemical sensors which rely on electrochemical reaction half-cell voltages or reaction currents, or measuring dimensional, mechanical or other 15 physicochemical changes in a sensor due to analyte sorption.

In one embodiment, where characteristic optical response signatures are employed as an analyte discriminating means, combinatorial polymer sensors may be coupled with fiber optic arrays, excitation light sources and optical detectors. In this embodiment, the sensors and sensor arrays of the present invention are typically fabricated by immobilizing a copolymer sensor at the end of an optical fiber or a 20 fiber optic array, comprised of either a fiber optic bundle, a preformed, unitary fiber optic array or imaging fiber comprising a plurality of individual fibers. In one preferred embodiment, a dye compound may be incorporated within a copolymer matrix which is photodeposited by photoinitiated copolymerization of two or more different monomers or oligomers. While solvatochromic dyes have particular utility in these sensor and sensor array embodiments, any indicator dye may be employed with such sensors to 25 produce a characteristic optical response upon exposure to excitation light in the presence of analytes.

Where dye compounds are employed in copolymer optical sensors and sensor arrays, solvatochromic dyes are particularly useful since these dyes are known to exhibit shifts in emission wavelength depending on the polarity of the local polymer matrix-analyte environment. When solvatochromic dyes are employed, an analyte contacting the copolymer sensor element typically alters the polymer 30 microenvironment, generally producing a change in polarity and giving rise to a complex temporal change in the fluorescence signal of the sensor when subject to excitation light energy. The phase, intensity, and shape of these temporal outputs depend directly on the physical and chemical nature of the particular polymer matrix in which the dye is entrapped.

While the copolymer sensors of the present invention may be employed as either analyte-specific 35 sensors or as analyte-specific sensor elements in a sensor array where specific sensor elements are utilized for detecting specific analytes, the sensors and sensor arrays of the present invention would

have particular utility as semi-selective sensor elements in cross-reactive sensor arrays. Semi-selective sensors are sensors which interact with a large number of analytes and do not interact exclusively with a specific analyte. Such sensors provide for a broad range of responses to a variety of analytes. That is, analytes can be detected and/or identified by a "signature" of individual responses on the sensors, similar to the manner in which "electronic noses" work. Cross-reactive sensor arrays are arrays which comprise a number of primary sensor elements which may either be selective to specific analytes or semi-selective to a variety of analytes. Typically, such arrays comprise a number of individual, broadly responsive sensor elements which are semiselective. The sensing elements of such arrays can provide a spatially distributed, time-varying response to a variety of analytes and are differentially responsive to a large number of analytes. The application of semi-selective sensor elements in cross-reactive sensor arrays thus enables using sensors and sensor materials which may not otherwise be useful as analyte specific sensors in conventional sensor array applications. The combinatorial polymer synthesis method for fabricating copolymer sensors of the present invention is particularly suitable for creating semi-selective sensors for these sensor applications. By providing new classes and families of copolymer sensors using the combinatorial polymer synthesis method of the present invention, expanded numbers of unique and diverse chemical sensors are thus provided for in a variety of sensor transduction mechanisms so as to increase the discriminating capabilities of chemical sensors and sensor arrays by increasing the diversity in the characteristic temporal responses of such sensors to a variety of analytes.

## 20 BRIEF DESCRIPTION OF THE DRAWINGS

This invention is pointed out with particularity in the appended claims. Other features and benefits of the invention can be more clearly understood with reference to the specification and the accompanying drawings in which:

Fig. 1 is a schematic diagram of the photodeposition system used for fabricating the copolymer sensors of Examples 1-3;

Fig. 2 is a schematic block diagram of the apparatus and instrumentation used for measuring the optical response characteristics of copolymer sensors of the present invention;

Fig. 3 is a fluorescence image of a sensor array comprising eight discrete PS802/MMA copolymer sensors of the present invention;

Figs. 4a-d compare the temporal fluorescence responses of eight discrete PS802/MMA copolymer sensors with various MMA additions upon exposure to a) benzene, b) hexane, c) 2-propanol, and d) ethyl acetate;

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Figs. 5a-b compare a typical fluorescence image of a PS802/PS901.5 gradient copolymer sensor of the present invention in Fig. 6a with a PS901.5 non-combinatorial polymer sensor in Fig. 6b;

Figs. 6a-b compare temporal fluorescence responses observed at consecutive points along a PS802/PS901.5 gradient copolymer sensor in Fig. 6a with the responses of a PS901.5 non-combinatorial polymer sensor in Fig. 6b upon exposure to benzene;

Fig. 7a-b compare temporal fluorescence responses observed at consecutive points along a PS802/PS901.5 gradient copolymer sensor in Fig. 7a with the responses of a PS901.5 non-combinatorial polymer sensor in Fig. 7b upon exposure to methanol;

Figs. 8a-b compare a fluorescence image of a PS802/MMA gradient copolymer sensor of the present invention in Fig. 8a with a PS802 non-combinatorial polymer sensor in Fig. 8b;

Figs. 9a-b compare temporal fluorescence responses observed at consecutive points along a PS802/MMA gradient copolymer sensor in Fig. 9a with the response of a PS802 noncombinatorial polymer sensor in Fig. 9b upon exposure to hexane;

Figs. 10a-b compare temporal fluorescence responses observed at consecutive points along a PS802/MMA gradient copolymer sensor in Fig. 10a with the response of a PS802 non-combinatorial polymer sensor in Fig. 10b upon exposure to methanol;

Figs. 11a-b compare temporal fluorescence responses observed at consecutive points along a PS802/MMA gradient copolymer sensor in Fig. 11a with the response of a PS802 non-combinatorial polymer sensor in Fig. 11b upon exposure to benzene;

Fig. 12 is a dendrogram plot comparing the dissimilarity in response of discrete copolymer sensors to benzene vapor; and

Fig. 13 is a multidimensional scaling plot comparing the dissimilarity in responses of a gradient copolymer sensors and control sensor.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

### 1. Combinatorial Synthesis of Copolymer Sensors

The copolymer sensor elements and sensor arrays of the present invention are fabricated by thermal polymerization, photopolymerization, crystallization or precipitation of from a precursor solution, comprising of a mixture of monomers or oligomers in varying compositional ratios, to form copolymers having distinguishing polymer structures. Particularly useful sensor candidates for use in the copolymer

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sensors and sensor arrays of the present invention are monomers, oligomers or prepolymers which, when polymerized, exhibit either characteristic swelling responses, characteristic polarity differences, characteristic electrochemical potentials, characteristic electrochemical currents, characteristic conductance, characteristic solvation effects, characteristic partition coefficients, characteristic sorption properties or characteristic structural features upon exposure to various analytes due to combinations of such materials in unique composition ratios. In selecting candidate monomers, oligomers, and copolymers as sensor materials and evaluating candidates based on desirable swelling, polarity, solvation, partition and adsorption characteristics, two particularly useful references are: R.A. McGill, et al., *Chemtech*, September 24, 1996, p 27-37 and J.W. Grate, et al., *Anal. Chem.* 68:913-7 (1996), hereby incorporated by reference.

The choice of copolymer sensor families used to form the sensor array elements in a particular sensor array is primarily determined based on the transduction mechanisms to be employed, the analytical purposes of the sensor, and the analytes which are anticipated targets for detection. Features such as polymer sensor matrix polarity, chemical structure, chemical functionality, surface area, pore size, swelling characteristics, partition coefficients, solvation properties, or chemical sorption behavior, either separately or in combination, contribute to the characteristic response signature of a given polymer sensor type. In one embodiment, sensor materials which are permeable or semi-permeable to vapor or liquid analytes are preferred. In another embodiment, sensor materials that swell upon contact with vapor or liquid analytes are preferred. In general, monomer, oligomer and polymer materials which have unique polarity; structure, pore size, surface area, functionality, analyte partitioning features or adsorption characteristics are particularly useful for copolymer sensor matrices of the present invention.

The combinatorial polymer method for fabricating sensors of the present invention can utilize polymerizable prepolymer mixtures of any monomers or oligomers that copolymerize by either photopolymerization or thermal polymerization and which are either themselves miscible or are miscible in a solvent. The method provides for a wide diversity of compositions selected from diverse polymer families such as vinyl or olefin polymers, including free radical polymers or addition polymers, and condensation polymers, where any combination or permutation of two or more distinct monomers or oligomers may be polymerized to form linear, branched or crosslinked copolymers. By utilizing either discrete compositional ratios or a varying compositional ratio gradient of two or more monomers or oligomers in the prepolymer mixture, copolymerized sensor matrices may be fabricated having unique, distinguishable, and characteristic swelling behavior, polarity, conductivity, resistivity, piezoelectric properties or chemical adsorption characteristics upon exposure to analytes.

#### A. Monomer or Oligomer Selection

A variety of polymer sensor chemistries may be utilized in fabricating a wide diversity of sensor families according to the method of the present invention. As will be appreciated by those in the art, while different sensors below list sets of suitable polymers, any of the monomer/polymer combinations may

be used for any sensor. By way of example, a monomer or oligomer may be selected from any member of the group of condensation polymers derived from such monomers as alcohols, dialcohols, amines, diamines, esters, diesters, carboxylic acids, dicarboxylic acids, diacid chlorides, carbonates, anhydrides, amides, imides, benzoxazoles, benzthiazoles, benzimidazoles, quinoxalines, aromatic compounds, including specific polymers such as phenol-formaldehydes, ureaformaldehydes, melamine-formaldehydes, acetyl compounds, lactones, nylons, or polyesters. Alternatively, a monomer may be selected from any member of the group of step-type reaction polymers comprising sulfones, ethers, phenylene oxides, phenylene oxide ethers, Diels-Alder-type reactants, urethanes and arylenes. Monomers may alternatively be selected from any member of the group of vinyl polymers comprising ethylenes, vinyl chlorides, vinylidene chlorides, tetrafluoroethylenes, acrylonitriles, acrylamides, acrylates, methacrylates, acetates, styrenes, including derivatized styrenes such as methyl styrenes, vinyl esters, vinyl pyrrolidones, butylenes and butadienes.

For optical sensors, sensor elements are typically selected based on distinguishable differences in their characteristic optical response signatures when illuminated by excitation light energy in the presence of a target analyte. In fabricating polymer sensor arrays, polymer sensor elements are selected which have characteristic optical response signatures when infiltrated with a reporting dye and illuminated by excitation light energy in the presence of a target analyte. Thus, preferred optical sensor materials for copolymer sensor arrays are selected based on both physical and chemical differences in sensor types which, in combination with a reporter dye compound, produce a characteristic optical response signature in the presence of the analyte when illuminated by excitation light energy.

The following monomer, polymer and copolymer compositions and their derivatives are particularly useful as candidate copolymer materials for combinatorial polymer optical sensors of the present invention: polyethylene glycol, polycaprolactone, polyarylamide, methyl methacrylate [MMA], 2-hydroxyethyl methacrylate, siloxane, dimethylsiloxane, acrylamide, methylenebisacrylamide [MBA], poly (1,4-butylene) adipate, poly (2,6-dimethyl-1,4-phenyleneoxide) [PDPO], triethoxysilyl-modified polybutadiene (50% in toluene) [PS078.5], diethoxymethylsilyl-modified polybutadiene in toluene [PS078.8], acryloxypropylmethyl- cyclosiloxane [CPS2067], (80-85%) dimethyl-(15-20%) (acryloxypropyl) methylsiloxane copolymer [PS802], poly(acryloxypropyl-methyl)siloxane [PS901.5], (97-98%) dimethyl-(2-3%) (methacryloxypropyl)methylsiloxane copolymer [PS851], poly(acrylonitrile-butadiene-styrene) [PABS], poly(methyl methacrylate), poly(styrene-acrylonitrile 75:25) [PSAN], acryloxypropylmethylsiloxane-dimethylsiloxane copolymer, methylstyrenes, styrenes, acrylic polymers, and methylstyrene divinyl benzene.

Copolymer electrical or electronic sensors which rely on conductance, inductance or resistance as a transduction means for detection of analytes may also be fabricated by the combinatorial polymer synthesis method of the present invention. The following monomer, polymer, and copolymer compositions and their derivatives would be particularly useful as combinatorial polymer candidate

materials for electrical conductive, inductive or resistive copolymer sensors and sensor arrays of the present invention: metallocene-based ethylenes, ethylene oxides, aromatics, alkyl or alkoxy substituted aromatics, dimethyl siloxanes, alkythiophenes, pyrroles, thiophenes, 3-methyl thiophene, 3,4-dimethyl thiophene, anilines, N-vinyl carbazole, p-phenylene vinylene, acetylenes, styrenes, p-phenylenes, 5 ethylenes, propylenes, dienes, vinyl chlorides, carbonates, vinyl acetates, urethanes, butadienes, acrylates, methacrylates, chloromethylated styrenes, vinylamines, and arsenic pentafluoride or iodine-doped combinatorial polymers comprising polyacetylene, poly-p-phenylene, polypyrrole and polyphenylene sulfide.

Copolymer piezoelectric sensor coatings which have distinguishable analyte sorption, solvation and 10 partition coefficient effects may also be fabricated by the combinatorial polymer synthesis method of the present invention. For example, thin films of the following monomer, polymer, and copolymer compositions and their derivatives are particularly useful as combinatorial polymer synthesis candidate coatings for piezoelectric substrates in piezoelectric-based sensor arrays of the present invention: polyimides, polycarbonates, phthalocyanines, styrene-butadiene-styrene copolymers, cyclophanes, 15 phthalocyanines, thiols, silanes, lipids, nucleic acids, enzymes, antibodies, triethanolamine, quadrol, ethylene dinitrotetraethanol, ascorbic acid, capiscum, L-glutamic acid, pyridoxine, triphenylamine, methyl-trioctylphosphonium dimethyl phosphate, glutathione, NAD, ethylene maleate, triethanolamine, vinyl stearates, collodion, butadiene-acrylonitriles, p-vinyl phenol, hydroxy terminated polybutadiene, vinyl isobutyl ethers, poly vinyl chlorides, caprolactones, caprolactone triols, butadiene methacrylate, methyl 20 methacrylates, polystyrenes, ethylene glycol methyl ether, vinyl carbazoles, abietic acid, octadecyl vinyl ether / maleic anhydride, polyethylenes, ethylcellulose, fluoropolyols, siloxanes, alkylaminopyridyl-substituted polysiloxanes, poly(4-vinyl hexafluorocumyl alcohol), hexafluor-2-propanol-substituted polysiloxanes, polyepichlorohydrin, polybis(cyanopropyl)-siloxanes, polyvinyl tetradecanals, polyisobutylenes, poly(trifluoropropyl)methylsiloxanes, polyethylene maleates, polyvinyl propionates, 25 polyethyl enimes, polyphenyl ethers, docosanol, diglycerols, polydiphenoxyposphazenes, diethyleneglycol adipate, polychloroprenes, acrylonitrile butadienes, apiezon L, biscyanoallyl polysiloxane, polyepichlorohydrin, phenylmethyldiphenylsilicone, vinyl-modified trifluoropropylmethylsilicone, tributoxyethyl phosphate, poly(hexyl arylate), poly(2-hydroxyethyl arylate), N-ethyl o,p-toluene sulfonamide, and phenyl ethers.

30 Copolymer sensors which demonstrate affinity for specific carbohydrates or sugars due to either hydrogen bonding or boron bonding, via ester bonds, to sugar molecules are also fabricated by the combinatorial polymer synthesis method of the present invention. In one embodiment, such sensors are produced by incorporating a number of different boronic acids, for example anthrylboronic acid or phenylboronic acid, in varying ratios into a polymer or reacting various boronic acids with a polymer, 35 thereby enabling the synthesis of copolymers having unique affinities for selectively binding specific sugars and carbohydrates.

Sensors made from the following hydrophilic monomer, oligomer, and prepolymer compositions and their derivatives are particularly useful as combinatorial polymer synthesis candidate sensor materials for sugars and carbohydrates in copolymer sensor arrays of the present invention: boronic esters, anthrylboronic acids, anthrylpolyamines, polyethylene glycols, polyalcohols, polyvinyl alcohols, 5 polyethers, polyethylene oxides, polyesters, poly HEMA, polyethylene terephthalate, polyamides, polyacrylamides, nylons, polycarboxylic acids, polyacrylic acid, or polymaleic acid.

Sensors made from copolymers of these compounds rely on affinity of sugars or carbohydrates for either hydrogen bonding or boron bonding with the copolymer. For copolymer sensors which rely on hydrogen bonding, the presence of *cis*-hydroxyl groups are particularly useful. Carbohydrate and sugar 10 sensors may be employed in sensor arrays which rely on detecting changes in mass due to analyte adsorption or, when combined with fluorescent dyes, they may be used in optical sensor arrays for detecting characteristic optic response signatures in response to analytes. Particularly useful candidate dye materials include fluorescent indicators which may be either incorporated into or conjugated with the copolymer sensor as discussed below.

15 Copolymer sensors which demonstrate affinity for specific metal ions or ion salts due ion-copolymer complex formation may also be fabricated by the combinatorial polymer synthesis method of the present invention. Charged ions having different affinities for metal ions and ion salts are combined with copolymers in varying combinations and ratios to generate unique sensor materials having unique affinities, binding properties and unique response characteristics to analytes. Copolymer sensors made 20 from the following hydrophilic monomer, oligomer, and prepolymer compositions and their derivatives would be particularly useful as candidate combinatorial polymer synthesis sensors for metal ions and ion salts in copolymer sensor arrays of the present invention: polyethylene glycols, polyalcohols, polyvinyl alcohols, polyethers, polyethylene oxides, polyesters, poly HEMA, polyethylene terephthalate, polyamides, polyacrylamides, nylons, polycarboxylic acids, polyacrylic acid, or polymaleic acid.

25 The affinity of candidate copolymer sensors for metal ions and ion salts may be modified by either negatively-charged ligands, such as carboxylic acids, sulfonic acids, phosphates and phosphonates, which attract metals or positively charged ions, or positively charged ligands, such as tetra alkylammonium and phosphonium salts, which attract ion salts or negatively charged ions. Such ligands may further modify the affinity of copolymer sensors by changes in ion coordination, charge repulsion, 30 or steric effects. These ion sensors may be employed in sensor arrays which either rely on detecting changes in mass due to analyte adsorption, or, when combined with fluorescent dyes in optical sensor arrays, detecting characteristic optic response signatures in response to analytes, or, in electrochemical sensor arrays, detecting characteristic potential, current or conductance ranges. Particularly useful candidate dye materials include metallochromic indicators, such as azo and triphenylmethane dyes, and 35 fluorescent indicators which may be incorporated into conjugated with the copolymer sensor as discussed below.

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Copolymer sensors which demonstrate characteristic hydrophilic or hydrophobic properties are also fabricated by the combinatorial polymer synthesis method of the present invention for detecting analytes by partitioning of target analytes between the copolymer and a fluid sample medium. With such sensors, a hydrophobic analyte would demonstrate affinity for a hydrophobic copolymer sensor in a hydrophilic fluid medium and hydrophilic analyte would demonstrate affinity for a hydrophilic copolymer sensor in a hydrophobic fluid medium. Alternatively, a hydrophobic copolymer repels a hydrophilic analyte and a hydrophilic copolymer repels a hydrophobic analyte. The relative affinity or repulsion of an analyte for a copolymer sensor leads to partitioning of the analyte between the copolymer and fluid medium and this characteristic may be employed for detecting an analyte in a fluid due to a characteristic physical, electrical, optical or chemical response of the sensor to the partitioned analyte. These analyte-partitioning copolymer sensors may be employed in sensor arrays which either rely on detecting changes in mass due to analyte adsorption from partitioning, or, when combined with fluorescent dyes in optical sensor arrays, detecting characteristic optic response signatures in response to partitioned analytes, or, in electrochemical sensor arrays, detecting characteristic potential, current or conductance changes due to partitioned analytes.

By way of example, copolymer sensors made from hydrophilic monomer, oligomer, and prepolymer compositions and their derivatives are particularly useful as combinatorial polymer synthesis candidate sensors for hydrophilic analytes such as metals, sugars, amino acids, amines, and carboxylic acids. Hydrophilic copolymer sensors made from the following hydrophilic monomer, oligomer, and prepolymer compositions and their derivatives are thus particularly useful as candidate sensors which rely on partitioning approaches: anthrylpolyamines, polyethylene glycols, polyalcohols, polyvinyl alcohols, polyethers, polyethylene oxides, polyesters, poly HEMA, polyethylene terephthalate, polyamides, polyacrylamides, nylons, polycarboxylic acids, polyacrylic acid, or polymaleic acid.

Alternatively, copolymer sensors made from hydrophobic monomer, oligomer, and prepolymer compositions and their derivatives are particularly useful as combinatorial polymer synthesis candidate sensors for hydrophobic analytes such as organic vapors or liquids, alkanes, alkenes, alkynes, alcohols, epoxides, polynuclear aromatic hydrocarbons (PAHs), polychlorobiphenyls (PCBs) and other hydrocarbons. Hydrophobic copolymer sensors made from hydrophobic monomer, oligomer, and prepolymer compositions and their derivatives containing the following functional groups are thus particularly useful as candidate sensors which rely on partitioning approaches: alkanes, alkenes, alkynes, alcohols, epoxides, aromatics, and polycyclic aromatics.

Additionally, copolymer sensors whose characteristic polymer structure creates steric impediments to analyte adsorption provide for partitioning of analytes due to size exclusion effects. By varying ratios of monomers or oligomers to crosslinkers in a prepolymer mixture, a diverse range of steric effects are introduced in a copolymer which provides for partitioning of analytes due to molecular size. By way of example, copolymer sensors based on poly HEMAs, polyacrylamides, as used in electrophoresis gels,



and cellulose acetates, as employed in dialysis membranes, are particularly useful oligomer candidates for copolymer sensors of the present invention where analytes are detected by size partitioning of target analytes between the copolymer sensor and a fluid sample medium. For vapor sensing, polystyrene with varying amounts of divinylbenzene may be employed to provide size exclusion of vapor analytes.

- 5 Examples of cross-linkers which are particularly useful for such copolymer sensors include ethylene glycol dimethacrylate (EGDMA) for poly HEMAs, methylene bisacrylamide with polyacrylamides, and divinylbenzene for polystyrene. Such sensors are particularly useful for detecting target analytes such as vapors, macromolecules or oligonucleotides such as proteins and DNA. With such copolymer sensors, the relative size of target analytes provides for partitioning of analytes between the copolymer
- 10 and fluid medium and this characteristic is employed for detecting such analytes in a fluid due to a characteristic physical, electrical, optical or chemical response of the sensor to the partitioned analyte. These analyte-partitioning copolymer sensors are employed in sensor arrays which either rely on detecting changes in mass due to analyte adsorption from partitioning, or, when combined with fluorescent dyes in optical sensor arrays, detecting characteristic optic response signatures in response
- 15 to partitioned analytes, or, in electrochemical sensor arrays, detecting characteristic potential, current or conductance changes due to partitioned analytes.

- Particularly useful candidate dye materials for either hydrophilic, hydrophobic or steric analyte-partitioning copolymer sensors discussed above include, for hydrophilic sensing environments, metallochromic indicators, such as azo and triphenylmethane dyes, and fluorescent indicators which
- 20 may be incorporated into or conjugated with copolymer sensors as discussed below. Particularly useful candidate dye materials for such analyte-partitioning copolymer sensors in hydrophobic sensing environments include solvatochromic dyes.

- Other polymers and copolymers having distinguishable and characteristic swelling behavior, polarity, conductivity, resistivity, piezoelectric properties or chemical adsorption characteristics are likely
- 25 candidate materials. Additional sensor matrix candidate materials include copolymers of the compounds listed in Table 7, Table 8 and Table 10 of U.S. Patent 5,512,490 to Walt, et al., which is incorporated herein by reference.

- In one alternative embodiment, the physical and chemical properties of copolymer sensor materials are further modified by the addition of plasticizers, including, but not limited to, tritolyl phosphate (TTP),
- 30 triphenyl phosphate (TTP) or dibutyl phthalate (DBP). In another alternative embodiment, copolymer sensor materials may be further modified by attaching a desirable chemical functional group to the sensor surface or applying either a surface treatment or coating to modify the characteristic sensor response to analytes.

- Where the addition of a particular chemical functionality to a copolymer sensor is desirable, examples
- 35 of surface chemistries which may be attached to sensor surfaces are amines, carboxylic acids,

aldehydes, aliphatic amines, amides, chloromethyl, hydrazide, hydroxyl, sulfate, sulfonate, aromatic and aromatic amine groups. Additional chemical functionality has been utilized with polymer sensors to produce sensor specificity for certain target analytes or to provide for a characteristic optical response to target analytes. Such functionalities include basic indicator chemistry sensors, enzyme-based  
5 sensors, immuno-based sensors and gene-sensors. Examples of such useful functionalities may be found in U.S. Patent Application Serial No. 08/851,203 to Walt, et al., filed on May 5, 1997, which is incorporated herein by reference.

#### B. Polymerization and Initiators

In fabricating copolymer sensors of the present invention, polymerization of prepolymer mixtures of  
10 desired monomer combinations may be achieved by thermal polymerization, condensation polymerization, photoinitiated polymerization, or either crystallization or precipitation from solution followed by annealing.

Thermal polymerization may be utilized either with or without the addition of an initiator. In one embodiment, initiators may be employed to control the rate of thermal polymerization. Since it is often  
15 desirable to carry out copolymerization of monomer mixtures at low temperature to prevent side reactions, the selection of thermal initiators is generally restricted to organic peroxides, such as dialkyl peroxides or diacylperoxides, organic hydroperoxides, azo compounds, such as azobisisobutyronitrile, and organometallic reagents, such as silver alkyls. Alternatively, thermal initiation may be accomplished by redox agents, for example, in aqueous solutions, a persulfate salt used in combination with a bisulfite  
20 ion reducing agent may form an intermediate sulfate radical ion and subsequent hydroxyl radical initiator. Similar redox reaction initiators may be used by combination of alkyl hydroperoxides and a reducing agent, such as ferrous ion. Additionally, some monomers, such as styrenes, undergo free-radical polymerization when heated or exposed to excitation light energy. Alternatively, anionic or cationic polymerization catalysts may also be employed.

25 In one embodiment, combinatorial polymer synthesis is accomplished by way of condensation polymerization. With this method, no initiator is required and polymerization occurs by way of direct reaction of desired monomers either in the presence or absence of a catalyst to stabilize a metastable intermediate.

In one preferred embodiment, photoinitiated polymerization is utilized. One advantage of  
30 photopolymerization is that it offers greater reaction control than thermal polymerization and enables spatial control of local polymerization reactions which can be restricted to regions illuminated by directed light energy. Photopolymerization may be conducted either with or without a specific photosensitizer initiator compound. For example, in the absence of a specific photosensitizer, many candidate monomer materials that can undergo chain reaction polymerization are susceptible to  
35 photopolymerization since the absorption of light produces free radicals or ions. Examples of such

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compounds are unsaturated monomers including, but not limited to, vinyl alkyl ketones, vinyl bromides, styrene and derivatives, methyl methacrylate and isobutylene.

In one alternative embodiment, a photosensitizer must be added to the prepolymer mixture of monomers for photopolymerization of the copolymer. Photosensitizers are compounds that absorb light in a desired region of the spectrum, typically ultraviolet or visible light, and subsequently dissociate into free radicals or transfer absorbed energy directly to a monomer. While some thermal initiators, such as azo compounds and peroxides are also photosensitizers, many alternative initiators are used as photosensitizers even though they do not dissociate thermally at useful temperatures. Examples of particularly useful photosensitizers are carbonyl compounds, such as acetone, biacetyl benzophenone benzoin, or  $\alpha$ -chloroacetone, condensed ring aromatics, such as anthracene, peroxides, such as t-butyl peroxide or hydrogen peroxide, organic sulfides, such as diphenyl disulfide or dibenzoyl disulfide, azo compounds, such as azoisopropane, azobisisobutyronitrile or aryldiazonium salts, halogen-containing compounds, such as chlorine, chloroform, carbon tetrachloride, bromotrichloromethane, bromoform or bromine, metal carbonyls, such as manganese pentacarbonyl and carbon tetrachloride or rhenium pentacarbonyl and carbon tetrachloride, and inorganic ions, such as  $\text{FeOH}^{+2}$  or  $\text{FeCl}_4$ . In one preferred embodiment, benzoin ethyl ether initiator was utilized.

### C. Dye Selection

For optical sensors which rely on light absorption and emission, the selection of chemical dye indicators is also important to the design and performance of the combinatorial copolymer sensors and sensor arrays of the present invention. An important characteristic of candidate dye materials for optical sensor elements is that they can be readily incorporated into copolymer sensor matrices and that, once incorporated into a copolymer matrix, their optical response characteristics are modified by the localized polymer microenvironment. In one embodiment, at least one dye is incorporated into the copolymer sensor matrix by way of entrapment. In an alternative embodiment, two or more dyes may be incorporated into the copolymer sensor matrix and peak intensity ratio for the dye pair may be used for providing a characteristic optical response signature for target analytes. In an alternative embodiment, conjugated dyes, such as acryloyl fluorescein and others, may be utilized where it is desirable to incorporate the dye directly into the copolymer sensor material by way of covalent bonding. Particularly useful references for selection of candidate dyes such as metallochromic indicators, including azo and triphenylmethane dyes, and fluorescent indicators, which may be either incorporated or conjugated with copolymer sensors of the present invention, is Indicators [E. Bishop (ed.), Pergamon Press (New York 1972)], and the 6th Edition of Molecular Probes Handbook by Richard P. Haugland, both of which are incorporated herein by reference.

While the reporter dye may be either a chromophore-type or a fluorophore-type, a fluorescent dye is preferred because the strength of the fluorescent signal typically provides a better signal-to-noise ratio and improves detection of target analytes. In the most preferred embodiment, polarity-sensitive dyes

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or solvatochromic dyes are utilized. Solvatochromic dyes are dyes whose absorption or emission spectra are sensitive to and altered by the polarity of their surrounding environment. Typically, these dyes exhibit a shift in peak emission wavelength due to a change in local polarity. Polarity changes which cause such wavelength shifts can be introduced by the copolymerized matrix used for a particular sensor family, by the presence of a target analyte, or by the combination of the copolymer matrix and analyte interaction with the dye. The change in polarity creates a characteristic optical response signature which is useful for detecting specific target analytes. One preferred solvatochromic dye is Nile Red, available from Eastman Kodak (Rochester, NY). Nile Red exhibits large shifts in its emission wavelength peak with changes in the local environment polarity. In addition, Nile Red is soluble in a wide range of solvents, is photochemically stable, and has a relatively strong fluorescence peak. Alternatively, other solvatochromic dyes such as Prodan, 6-propionyl-2-(N,N-dimethylamino)naphthalene, or Acrylodan, 6-acryloyl(dimethylamino)naphthalene, available from Molecular Probes (Eugene, OR), may be employed. Additional dyes which are conventionally known in the art and may be used as dyes in the present invention are those found in U.S. Patent 5,512,490 to Walt, et al., of which Table 3, Table 4, Table 5, Table 6 and Table 11 are incorporated herein by reference.

Diverse families and types of optical sensor elements may be fabricated as sensors and sensor arrays of the present invention by incorporating reporter dyes, such as metallochromic indicators, fluorescent indicators, or solvatochromic dyes, within various copolymer matrices of varying monomer compositions. By incorporating such dyes in sensor elements made from different copolymer matrices of varying polarity, hydrophobicity, pore size, flexibility and swelling tendency, unique sensors are produced that react differently with molecules of individual analytes, giving rise to distinguishable and characteristic fluorescence responses when exposed to target analytes.

#### D. Sensor Configuration

The sensors of the present invention can be configured in a variety of ways, including, but not limited to, polymerization of the copolymer sensors directly on a substrate, polymerization of the copolymer sensors onto the surface of microspheres, and polymerization of the copolymer sensors into microspheres; the latter two embodiments then distribute the microspheres on the substrate.

In a preferred embodiment, the combinatorial copolymer sensors are polymerized directly onto discrete sites on a substrate. By "discrete sites" is meant individual sites or loci on a substrate. The sites may be a pattern, i.e. a regular design or configuration, or randomly distributed. A preferred embodiment utilizes a regular pattern of sites such that the sites may be addressed in the X-Y coordinate plane. A pattern in this sense includes a repeating unit cell, preferably one that allows a high density of sites on the substrate. However, it should be noted that these sites may not be discrete sites. That is, it is possible to use a uniform surface of adhesive or chemical functionalities, for example, that allows the association of copolymers, or in the case where microspheres are used, for association of the beads at any position. That is, the surface of the substrate is modified to allow association of the copolymers

(microspheres) at individual sites, whether or not those sites are contiguous or non-contiguous with other sites. Thus, the surface of the substrate may be modified such that discrete sites are formed that can only have a single associated copolymer, or alternatively, the surface of the substrate is modified and copolymers may go down anywhere, but they end up at discrete sites.

- 5 By "substrate" or "solid support" or other grammatical equivalents herein is meant any material that can be modified to contain discrete individual sites. In a preferred embodiment such sites are appropriate for the attachment or association of beads. The substrate also is amenable to at least one detection method. As will be appreciated by those in the art, the number of possible substrates is very large. Possible substrates include, but are not limited to, glass and modified or functionalized glass, plastics
- 10 (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, Teflon, etc.), polysaccharides, nylon or nitrocellulose, resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses, plastics, optical fiber bundles, and a variety of other polymers. Also, metals, ceramics, graphites, semiconductors, and composites fabricated from such materials are useful as substrates. In
- 15 general, the substrates allow optical detection and do not themselves appreciably fluoresce.

Such substrate materials may be preconditioned or functionalized using conventionally known surface treatments so as to improve sensor adhesion or to immobilize sensors on the substrate. The ultimate choice of a preferred substrate material depends on the actual sensor format and transduction mechanism as well as the targeted sensing environment and application.

- 20 For electrical sensors, suitable substrates include conventional dielectric, magnetic, conducting, and semiconducting materials as well as electrical components such as electrodes, capacitors, resistors, diodes and transistors. For mass sensitive sensors, potential substrates include any piezoelectric materials or devices such as quartz crystal microbalances, microcantilevers, and surface acoustic wave devices. For surface plasmon resonance sensors, potential substrates include thin reflective surface
- 25 films comprised of gold, silver, chrome, nickel or any other highly reflective material. For optical sensors, transparent, opaque or reflective substrates may be employed depending on the orientation of the sensor relative to the analyte medium and incident excitation light source, and detection means. In one preferred embodiment, an individual fiber optic strand or a fiber optic array, comprising either a fiber optic bundle, or preformed, unitary fiber optic array, or an imaging fiber, comprised of a plurality of
- 30 individual fibers, may be used as an optical sensor substrate where the excitation light and optical response of the sensor are conveyed to and from sensors which are immobilized on one end of the fiber strand or fiber array.

- Accordingly, a preferred embodiment utilizes fiber optic sensors. This is particularly useful when photoinitiation is done. As one of ordinary skill in the art appreciates, when using fiber optic bundles, light
- 35 is transmitted through individual fibers where it contacts the copolymer thereby initiating polymerization.

From one to all of the individual fibers of a fiber optic bundle are illuminated. In an alternative embodiment, only a subset of fibers are illuminated.

In a preferred embodiment, the sensors of the invention comprise microspheres. By "microspheres" or "beads" or "particles" or grammatical equivalents herein is meant small discrete particles. The composition of the beads will vary, depending on the configuration of the array and the copolymers used, as well as the method of synthesis. Suitable bead compositions include those used in peptide, nucleic acid and organic moiety synthesis, including, but not limited to, plastics, ceramics, glass, polystyrene, methylstyrene, acrylic polymers, paramagnetic materials, thoria sol, carbon graphite, titanium dioxide, latex or cross-linked dextrans such as Sepharose, cellulose, nylon, cross-linked micelles and Teflon may all be used. *"Microsphere Detection Guide"* from Bangs Laboratories, Fishers IN is a helpful guide.

The beads need not be spherical; irregular particles may be used. In addition, the beads may be porous, thus increasing the surface area of the bead available for exposure to the target analyte or for either bioactive agent attachment or IBL attachment. The bead sizes range from nanometers, i.e. 100 nm, to millimeters, i.e. 1 mm, with beads from about 0.2 micron to about 200 microns being preferred, and from about 0.5 to about 5 micron being particularly preferred, although in some embodiments smaller beads may be used.

It should be noted that a key component of the invention is the use of a substrate/bead pairing that allows the association or attachment of the beads at discrete sites on the surface of the substrate, such that the beads do not move during the course of the assay.

In a preferred embodiment, the copolymers are polymerized onto the surface of the beads. This may be desirable to increase the surface area, particularly when porous beads are used. Accordingly, each bead is contacted with a different combination of copolymers which are then polymerized onto the surface of the bead. This results in the formation of a population of heterogeneous bead sensors. Alternatively, each bead is contacted with the same combination of copolymers which are polymerized to create a homogenous population of bead sensors.

In a preferred embodiment, the copolymers are polymerized into beads that are then distributed on a surface. For example, any of the copolymer combinations are used to fabricate beads in accordance with methods known to those of ordinary skill in the art.

#### E. Substrate Selection

The combinatorial copolymer sensors of the present invention may be deployed on virtually any solid substrate material as either discrete individual sensors or as a plurality of sensor elements in a sensor array. Suitable substrate materials include, but are not necessarily limited to metals, ceramics, glasses, plastics, polymers, graphites, semiconductors, and composites fabricated from such materials. Such

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substrate materials are preconditioned or functionalized using conventionally known surface treatments so as to improve sensor adhesion or to immobilize sensors on the substrate. The ultimate choice of a preferred substrate material depends on the actual sensor format and transduction mechanism as well as the targeted sensing environment and application.

- 5 For electrical sensors, suitable substrates include conventional dielectric, magnetic, conducting, and semiconducting materials as well as electrical components such as electrodes, capacitors, resistors, diodes and transistors. For mass sensitive sensors, potential substrates include any piezoelectric materials or devices such as quartz crystal microbalances, microcantilevers, and surface acoustic wave devices. For surface plasmon resonance sensors, potential substrates include thin reflective surface
- 10 films comprised of gold, silver, chrome, nickel or any other highly reflective material. For optical sensors, transparent, opaque or reflective substrates are employed depending on the orientation of the sensor relative to the analyte medium and incident excitation light source, and detection means. In one preferred embodiment, an individual fiber optic strand or a fiber optic array, comprising either a fiber optic bundle, or preformed, unitary fiber optic array, or an imaging fiber, comprised of a plurality of
- 15 individual fibers, may be used as an optical sensor substrate where the excitation light and optical response of the sensor are conveyed to and from sensors which are immobilized on one end of the fiber strand or fiber array.

## 2. Sensor and Sensor Array Fabrication

### A. Discrete Copolymer Sensors

- 20 For fabrication of sensor arrays comprising a selection of discrete copolymer sensors formed by the method of the present invention, at least two or more monomers or prepolymers are employed at preselected monomer or prepolymer ratios so as to provide a broad range of monomer or prepolymer compositions. In one embodiment, an (80-85%) dimethyl-(15-20%)(acryloxypropyl)methylsiloxane copolymer, commonly known as PS802 and available from Gelest Inc. (Tullytown, PA), and methyl
- 25 methacrylate, commonly known as MMA and available from Aldrich (Milwaukee, WI) were selected as example monomers for copolymerization. Typically, separate solutions of monomer or prepolymer mixtures are prepared representing a range of monomer or prepolymer composition ratios. In one embodiment, the range of at least one monomer or prepolymer composition varies from between 0 to 100% of the monomer. In an alternative embodiment, at least one composition ranges from between
- 30 0 to 50%. In alternative embodiments, at least one composition ranges from between 0 to 25%. Other, more narrow, composition ranges may also be employed.

- The monomer or prepolymer mixtures are copolymerized on any suitable substrate (or into beads) by either a photoinitiated polymerization or a conventional thermal polymerization method. With either method, an initiator solution and dye solution are added to the prepolymer or monomer mixture either
- 35 prior to, during or after polymerization for the purpose of minimizing exposure and controlling reaction with the initiator. In a preferred embodiment, a solvatochromic dye is utilized. In one preferred

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embodiment, a solution of Nile Red in chloroform is used as the dye solution and a solution of benzoin ethyl ether in toluene is used as a photoinitiator.

In one preferred embodiment, each of the monomer mixture-dye-initiator solutions is individually photopolymerized and photodeposited onto the end of a preformed fiber optic array or imaging fiber.

- 5 In this embodiment, a distal end of the fiber is either immersed directly in a prepolymer solution or, alternatively, a polymerization solution is applied to a distal end of the fiber. Using conventional optical train components, a series of pinholes, lenses and objectives focuses an ultraviolet light beam on pre-selected fiber end faces of the fiber array. The light is transmitted down the length of the individual fibers and exits at the distal end faces of the fibers at the predetermined fiber locations in the array. The
- 10 photopolymerization of individual prepolymer monomer mixtures is thereby initiated at designated regions across the distal end surface of the fiber array. In this manner, the photodeposition method and system described herein provides for the formation of a plurality of discrete sensor elements and sensing regions across the distal end surface of the fiber array.

- Typically, the size of the photopolymerized deposit may be controlled by the polymerization reaction time
- 15 and polymerization rate. The reaction rate is influenced by polymerization light intensity, concentrations of photoinitiator and monomers and choice of initiator and monomer composition. The size of photodeposits can be controlled over a diameter range from several microns, covering the end of an individual fiber strand in the fiber optic array, to over 100 microns, covering adjacent multiple fiber strands. In one embodiment, each of the prepolymer monomer mixture solutions was polymerized for
- 20 5 seconds, resulting in approximately 45 $\mu$ m-diameter polymer hemispheres or cones attached to the distal end of a fiber optic array. Multiple deposits of each monomer combination mixture may be polymerized at various fiber locations in the fiber optic array. By forming multiple deposits of each monomer combination, a sensor array having a unique arrangement of sensing elements is thereby produced.

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#### B. Copolymer Gradient Sensors

- With the copolymer gradient sensors and gradient sensor arrays of the present invention, the elements of the sensor array are no longer discrete individual polymer deposits, but rather user-defined, regions-of-interest (ROI), outlining specific portions of the gradient polymer deposit to be monitored. Following the collection of a sequence of fluorescence image frames with a CCD camera, one can use
- 30 standard drawing tools included in commercial image processing software packages to either manually or automatically select areas of the gradient sensor images from which to measure fluorescence. Examples of such pre-selected areas are shown in Figs. 5a-b and Figs. 8a-b.

- Typically, copolymer gradient sensors are prepared by photodepositing a polymer strip from a prepolymer mixture having a time-varying monomer composition. In a typical procedure, the distal end
- 35 of a fiber optic array is immersed in a stirred first solution comprising at least one monomer and a



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photoinitiator. For photodeposition of the copolymer, a UV excitation light beam is scanned across the proximal end of the fiber array at a predetermined scanning rate while a second solution, comprising at least one additional monomer and a photoinitiator, is simultaneously added and mixed with the first solution. Generally, both solutions are flushed with nitrogen prior to the mixing photopolymerization of the monomers. This photodeposition method produces a copolymerized polymer strip having a continuously varying structure and composition over its entire length due to the monomer composition variation imposed in the prepolymer mixture during polymerization of the solution. After photodeposition of the copolymer gradient strip, the deposit is soaked in a dye solution comprising a solvent and dye, rinsed, and dried overnight. In a preferred embodiment, the copolymer gradient sensors are contacted with a dye solution comprising solvatochromic dye dissolved in an organic solvent that will swell the copolymer gradient sensor matrices. In a subsequent step, the gradient sensors are washed to remove excess dye. Typically, the sensors are washed in water, methanol, or any suitable solvent that does not swell the copolymer matrix, but in which the dyes are still soluble. This allows the residual dye to be rinsed off without rinsing the dye out of the sensor matrices.

#### 15 C. Sensor Array Fabrication

The copolymer sensor elements of the present invention may be deposited on any suitable substrate materials to form a sensor or sensor array. A wide diversity of materials may be utilized as substrates. While the substrate may be formed from any suitable organic or inorganic materials, the substrate should be chemically inert to the sensor elements, target analytes and any analyte solvent matrix. Examples of suitable substrate materials include glass, ceramics, plastics, polymeric materials, metals or composite materials. The size, shape, and configuration of the substrate can be adapted to meet the requirements of a particular sensing application or environment. A principal requirement in selecting a suitable substrate dimension and configuration is that the substrate should provide for access of target analytes to the copolymer sensor elements. The variety of substrate configurations and shapes envisioned by the sensor of the present invention includes fibers, rods, plates, spheres, or any curved, rectilinear or irregular surfaces as well as piezoelectric substrates and electrodes.

In one embodiment for optical sensor arrays, a sensor array 100, comprising a plurality of individual sensors, are located adjacent to or attached to a distal end 212 of an optical fiber bundle 202. In one preferred embodiment, discrete sensors or gradient sensors are deposited directly on a distal end 212 of a fiber bundle 202. It is important that the sensors and sensor array are located proximate to the distal end 212 of the fiber optic bundle 202 to ensure that the light returning in each discrete optical fiber 252 predominantly originates from only a single sensor or a portion of a gradient sensor. This feature is necessary to enable the interrogation of the optical signature of individual sensors or portions of gradient sensors within the sensor array 100, and to provide for the summing of individual sensor responses within each sensor type for reducing signal to noise and improving signal enhancement. In this embodiment, the sensor adhesion or affixing technique must not chemically insulate the sensor from the analyte or otherwise interfere with the optical measurement.

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In one preferred embodiment, each individual optical fiber 252 of the fiber bundle 202 conveys light from a single discrete sensor or a portion of a gradient sensor.

Consequently, by imaging the light emanating from a proximal end 214 of the fiber bundle 202 onto a detector array such as a CCD camera, the optical signatures of discrete sensors or portions of gradient  
5 sensors are individually interrogatable.

Where a fiber optic array is employed as a sensor array 100 substrate, the fiber optic array may be either a conventional imaging fiber, comprising a preformed, unitary array of a plurality of prefused individual fibers, or a conventional fiber bundle comprised of a plurality of discrete individual fibers, where the individual fiber strands are disposed coaxially along their lengths. In one preferred  
10 embodiment, the individual fibers have a cladding for reducing optical crosstalk between fibers in the array. While a fiber optic array will typically comprise thousands of discrete fibers of uniform diameter, alternative embodiments can provide for variations in both the number of fibers and range of fiber diameters within an array. Individual fiber diameters of the fiber optic array may range from approximately 1 to 500  $\mu\text{m}$ . Individual fibers may have either a circular or non-circular cross-section and  
15 mixtures of cross-sections may be employed in an array. The individual fibers of the array may be arranged either in coherent or incoherent manner. For the sensor arrays described in Examples 1-3, commercial image guides were used as fiber optic array substrates for photodeposition of the copolymer sensor elements. These image guides, available from Galileo Electro-Optics Corporation (Sturbridge, MA), typically comprise approximately 6000 optical fibers, each fiber having a 2-4  $\mu\text{m}$  diameter, packed  
20 together in a coherent fashion such that spatial position is maintained from one end of the fiber to the other.

Prior to photodeposition of copolymer sensor elements, the distal end surface of the fiber optic array is typically cut to length, polished and prepared for photodeposition. Generally, both the proximal and distal ends of the fiber array bundle are successively polished on 12  $\mu\text{m}$ , 9  $\mu\text{m}$ , 3  $\mu\text{m}$ , 1  $\mu\text{m}$ , and 0.3  $\mu\text{m}$   
25 lapping films. Subsequently, the ends may be inspected for scratches on a conventional microscope. The fiber is typically rinsed in water and acetone and ultrasonically cleaned for several minutes to remove any polishing residue. The fiber is then allowed to dry prior to a silanization treatment.

When the sensor comprises beads, the substrate can be prepared in a variety of ways. In a preferred embodiment, when the sensor comprises beads, the substrate may be prepared to include discrete  
30 sites. In a preferred embodiment, the surface of the substrate is modified to contain wells, i.e. depressions in the surface of the substrate. This may be done as is generally known in the art using a variety of techniques, including, but not limited to, photolithography, stamping techniques, molding techniques and microetching techniques. As will be appreciated by those in the art, the technique used will depend on the composition and shape of the substrate.

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In a preferred embodiment, physical alterations are made in a surface of the substrate to produce the sites. In a preferred embodiment, the substrate is a fiber optic bundle and the surface of the substrate is a terminal end of the fiber bundle, as is generally described in 08/818,199 and 09/151,877, both of which are hereby expressly incorporated by reference. In this embodiment, wells are made in a terminal  
5 or distal end of a fiber optic bundle comprising individual fibers. In this embodiment, the cores of the individual fibers are etched, with respect to the cladding, such that small wells or depressions are formed at one end of the fibers. The required depth of the wells will depend on the size of the beads to be added to the wells.

Generally in this embodiment, the microspheres are non-covalently associated in the wells, although  
10 the wells may additionally be chemically functionalized as is generally described below, cross-linking agents may be used, or a physical barrier may be used, i.e. a film or membrane over the beads.

In a preferred embodiment, the surface of the substrate is modified to contain chemically modified sites, that can be used to associate, either covalently or non-covalently, the microspheres of the invention to the discrete sites or locations on the substrate. "Chemically modified sites" in this context includes, but  
15 is not limited to, the addition of a pattern of chemical functional groups including amino groups, carboxy groups, oxo groups and thiol groups, that can be used to covalently attach microspheres, which generally also contain corresponding reactive functional groups; the addition of a pattern of adhesive that can be used to bind the microspheres (either by prior chemical functionalization for the addition of the adhesive or direct addition of the adhesive); the addition of a pattern of charged groups (similar to  
20 the chemical functionalities) for the electrostatic association of the microspheres, i.e. when the microspheres comprise charged groups opposite to the sites; the addition of a pattern of chemical functional groups that renders the sites differentially hydrophobic or hydrophilic, such that the addition of similarly hydrophobic or hydrophilic microspheres under suitable experimental conditions will result in association of the microspheres to the sites on the basis of hydroaffinity. For example, the use of  
25 hydrophobic sites with hydrophobic beads, in an aqueous system, drives the association of the beads preferentially onto the sites. As outlined above, "pattern" in this sense includes the use of a uniform treatment of the surface to allow association of the beads at discrete sites, as well as treatment of the surface resulting in discrete sites. As will be appreciated by those in the art, this may be accomplished in a variety of ways.

30 The copolymer sensor elements may be deposited with or without pretreating the fiber optic array end surface. In a preferred embodiment, the distal end surface of a fiber optic array bundle is typically activated with a silanizing reagent for improving the adhesion of the copolymer sensor deposit to the fiber array. For photodeposition of copolymer sensor elements, fiber array ends are typically treated by immersion in a 10% solution of 3(trimethoxysilyl)propyl-methacrylate in acetone, rinsed and cured in the  
35 absence of light for approximately one hour at room temperature. For thermal polymerization of copolymer sensor elements, the distal end of a fiber array is typically treated with a 2% solution of

noctadecyltriethoxysilane in 95% ethanol-acetic acid mixture at pH 4.5 for two minutes. The fiber end is rinsed with ethanol and cured at 100°C for 10 minutes.

For photodeposition of the copolymer sensor elements, in one embodiment the distal end of the fiber array may either be immersed in a prepolymer solution comprising a photoinitiator, dye, and at least two monomers that can be photopolymerized. Alternatively, the prepolymer solution may be applied to the distal end of the fiber. In one embodiment, a solvent may be utilized for improving the solubility of reagents in the prepolymer mixture. The proximal end of the fiber array is illuminated with excitation light at a predetermined intensity for a specified time depending on the composition and reactivity of the prepolymer solution. The time and intensity of light exposure is established by shutter and radiometer settings on the photodeposition system. In one embodiment, the fiber is illuminated while the distal end is immersed in the prepolymer solution. In an alternative embodiment, the fiber is dipped in the solution, removed and then illuminated. As a result of controlled illumination, a photodeposit of appropriate size and area is formed on the distal end of the fiber array. The photodeposition conditions may be controlled to produce precisely scaled, individual deposits on the end of each fiber in the fiber array or, alternatively, to produce a larger deposit which covers the ends of multiple adjacent fibers. Typically, for gradient sensor fabrication, a larger deposit encompassing multiple fiber ends is employed so that multiple regions of interest within the gradient sensor can be optically coupled to individual fibers in the fiber array.

In an alternative embodiment, dye may be incorporated into individual copolymer sensor elements or a sensor array following photodeposition of the copolymer matrix. With this embodiment, the individual sensors or, alternatively, the entire sensor array may be exposed to a dye solution comprised of a dye and appropriate solvent. For example, the copolymer sensors or sensor array may be contacted with a dye solution comprising solvatochromic dye dissolved in an organic solvent that will swell the copolymer gradient sensor matrices. In a subsequent step, the gradient sensors are washed to remove excess dye. Typically, the sensors are washed in water, methanol, or any suitable solvent that does not swell the copolymer matrix, but in which the dyes are still soluble. This allows the residual dye to be rinsed off without rinsing the dye out of the sensor matrices.

In one alternative embodiment, a chemical moiety or functional group may be attached to copolymer sensor elements either prior to the dye incorporation step or following removal of excess dye after the incorporation step.

#### D. Photodeposition System

Photodeposition of the discrete copolymer sensors and gradient copolymer sensors was performed with a modified commercial photodeposition system. A schematic diagram of the photodeposition system 50 is shown in Fig. 1. A Novacure ultraviolet spot-cure photodeposition system, available from EFOS (Ontario, Canada) was modified for the photopolymerization copolymer depositions of Examples 1

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through 3. A 100 Watt short-arc mercury-xenon lamp 105 with a liquid light guide was employed as a source for uv excitation light. The spot-cure system has a built-in radiometer for controlling the light intensity and an electronic shutter 110 for controlling the polymerization time. Typically, excitation light at 330 nm wavelength and approximately 1100mW/cm<sup>2</sup> intensity was utilized. Other wavelengths and intensities may be utilized to match specific monomer compositions and concentrations. The excitation light was collimated with a 50 mm focal length lens 115, passed through a series of neutral density filters 140, excitation filters 145, a 100 um pinhole 120, and focused onto the proximal end 214 of a fiber optic bundle 202 with a 15x reflecting microscope objective 125. A conventional fiber chuck 130 and a Spindler and Hoyer (Milford, MA) coarse xy-micropositioner 135 was utilized for securing, positioning and focusing the proximal end surface 214 of the fiber optic bundle 202 with respect to the excitation light beam. The xy-positioner 135 provided 360° rotation, allowing for precise positioning of the proximal end surface 214 of the fiber bundle 202. The distal end 212 of the fiber bundle 202 was immersed in the prepolymer solution 150 of monomers, photoinitiator and dye.

For copolymer gradient sensor depositions, a Burleigh Instruments (Fishers, NY) Model No. 6000 ULN Inchworm PZT nanopositioner 136 was utilized in conjunction with the coarse xy-positioner 135 and fiber chuck 130 for securing, positioning, and translating selected regions of the proximal end 214 of the fiber optic array 202 across the optical axis of a focussed light beam. The focussed light beam remained stationary while the proximal end surface 214 of the fiber bundle 202 was translated across the beam focal point using the nanopositioner 136. A hand-held controller provided precise remote control of the nanopositioner 136 scanning. Scanning speed was adjusted to accommodate either the polymerization rate or addition mixing rate of the monomers in the prepolymer solution 150 at the distal end 212 of the fiber bundle 202. In an alternate embodiment, the focussed light beam may be translated across a stationary proximal end 214 of the fiber bundle 202. While this modification of the photodeposition apparatus 50 provides for a single copolymer gradient sensor to be deposited at a time, it is anticipated that multiple copolymer gradient sensors can be fabricated either sequentially, by repositioning the proximal end surface 214 of the fiber optic array 202 with respect to the optical axis of the light beam, or simultaneously, using modifications of the optical train and apparatus to provide for multiple excitation light beams to be focused simultaneously on multiple areas of the proximal end surface 214 of the fiber array 202.

### 3. Experimental Measurements

#### A. Sensing Apparatus and System:

Characteristic temporal optical response data measurements of discrete copolymer sensors and selected regions of copolymer gradient sensors responses to specific vapor analytes and excitation light energy were made according to the general method, apparatus and instrumentation disclosed by White, et al., *Anal. Chem.* 68:2191-2202(1996), hereby expressly incorporated by reference.

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In Fig. 2, a schematic block diagram illustrates the experimental apparatus and instrumentation 200 used for data measurements obtained with fiber optic sensor arrays of the present invention. In a typical measurement, a proximal end 214 of a fiber optic array 202 was placed in a fiber chuck 210 and secured for viewing with an optical instrumentation system utilizing a modified Olympus microscope 220.

5 A sensor array 100 comprising a plurality of discrete sensors or gradient sensors disposed on a distal end 212 of the bundle 202 was exposed to a fluid sample containing analytes to be detected. In an alternative embodiment, a microscope 220 slide platform and slide clamp was used for viewing and positioning sensor array substrates, such as glass plates, slides or cover slips. The microscope 220 was equipped with an epi-illuminator ( $\lambda_{\text{ex}}$  540 nm,  $\lambda_{\text{em}}$  590 nm, Zeiss 2.5 objective, 0.8 NA) and Olympus

10 20x and 40x and Zeiss 100x objectives. An Omega 560 DCRP dichroic mirror 230 was used to direct excitation light energy from a 75W Xenon arc lamp 240, via the fiber bundle 202, to the sensor array 100 at the distal end 212 of the bundle 202. The dichroic mirror 230 was capable of reflecting light at shorter wavelengths (<560 nm) and transmitting light at longer wavelengths (>595 nm). The excitation light energy emanating from the arc lamp 240 was filtered by an Omega 535 BP40 integrated excitation light

15 filter/shutter 250. The characteristic optical response signature of the array 100, emitted by individual sensor elements or portions of gradient sensors upon illumination by excitation light in the presence of analytes, was transmitted via the fiber bundle 202 and dichroic mirror 230 to a CCD frame transfer camera 270. The light energy emitted from the sensors or portions of gradient sensor of the sensor array 100 was filtered with an Omega 640 BP20 integrated emitted light filter/shutter 260 prior to the

20 CCD frame transfer camera 270. In one embodiment a TE/CCD-512EFT Princeton Instruments (Trenton, NJ) 512 x 512 frame transfer CCD camera 236 was utilized for capturing frames of characteristic fluorescence response images of sensor elements in the sensor array 100.

A 8100AV Macintosh Power PC desktop computer 238 with a Princeton Instruments NUBus camera interface card was employed for data acquisition and processing images acquired by the CCD camera

25 236. Experiments generally consisted of collecting video camera frames of fluorescence response images and recording the images with the CCD frame transfer camera 270. Captured images are then conveyed to the camera interface card in the computer system 280. Depending on the response time of sensor elements, the duration of the analyte-sensor array interaction and the rise and decay of sensor responses to an analyte exposure, camera frame rates and measurement times are selected for

30 collection of a suitable number of data points. Frame capture times typically range between 80 to 250 ms/frame.

#### B. Sensor Response Testing:

A conventional air dilution olfactometer and vacuum-controlled vapor delivery system 290, as commonly known and used in olfactory research and described in Kauer, et al., *J. Physiol.* 272:495-516 (1977) and

35 White, et al., *Anal. Chem.* 68(13):2191 (1996) was used to apply controlled pulses of analyte vapor and air carrier gas to either a sensor substrate or the distal end 212 of a fiber optic sensor array 100 containing an array of discrete sensors or portions of gradient sensors.

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To produce a saturated vapor sample, in one embodiment, a stream of air carrier gas was passed through a 5 ml cartridge containing filter paper saturated with an analyte. In an alternative embodiment, the carrier gas was passed through a 100 ml sample of analyte contained in a 250 ml Erlenmeyer flask. Analyte dilutions were produced by adjusting the relative flow rates of saturated vapor and clean carrier gas streams. Typically, a flow rate of 150 ml/min is used for the combined gas flow to the sensor array. At this flow rate, a 3 second pulse would deliver approximately 7.5 ml of analyte vapor with carrier gas. In general, depending on the analyte vapor pressure and dilution factor, vapor pulses contain between  $1.6 \times 10^{-6}$  to  $4.0 \times 10^{-5}$  mol of analyte.

For 60 frame measurements, the vapor pulse is typically delivered during the 11th through 30th frame, commencing on the 11th frame. For 40 frame measurements, the vapor pulse is typically delivered during the 6th through 20th frame, commencing on the 6th frame. The duration of the vapor pulse varies with the specific frame rate utilized and typically ranges between 1 to 4 seconds. Baseline control measurements are performed with high purity, Ultra Zero grade air. The air pulse measurements are performed to account for any sensor responses due to the vapor carrier gas.

The sensor arrays of Example 1 and Example 2 were tested by exposing each array to a number of representative analyte vapors using the vapor delivery system and imaging system described herein. In typical sensor response tests for each analyte, a total of 40 time points were collected over 4 seconds, with a vapor pulse duration of 1s. The CCD camera integration time was set to 25ms, and pixels were binned 5 x 5 to enhance signal and reduce readout times. For testing these sensors, the sensor array was typically illuminated with 535nm light, and emission was monitored at 629nm using a 20 nm band pass, liquid crystal tunable filter (Cambridge Research Instruments, Cambridge, MA).

#### 4. Data processing and Analysis

##### A. Data Processing

Following the collection of a temporal series of sensor element or sensor array images, segments are typically drawn, using IPLab image processing software (Signal Analytics, Vienna, VA), over each pixel or groups of pixels which correspond to an individual fiber where the fiber is coupled to either a discrete sensor or a portion of a gradient sensor at its distal end. The mean fluorescence intensity is measured for each one of these segments in each frame in the sequence. This is done for both the vapor pulse responses and the baseline air pulse responses. Averages of multiple runs of each may be performed to improve data quality where needed. The air pulse data are then subtracted from the vapor pulse data to subtract the background due to air alone. The resulting data can be plotted to yield temporal intensity responses for all sensor types of interest.

All data manipulation is performed within the IPLab program environment using simple scripts written by the operator which call imbedded image or data processing functions. These scripts and routines consist of a data collection portion and a data analysis portion.

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In the data collection portion, there are three segments or loops as follows:

Loop 1. This establishes the baseline fluorescence of each sensor. This loop can be shortened or extended to adjust to slower or faster response times of specific sensors or sensor arrays to certain analytes. In typical measurement runs, this loop is set between 5 to 20 frames.

- 5 Loop 2. This is the vapor exposure loop. A vapor pulse is applied just before this loop starts by way of a script command that sends a 5 volt pulse to an attached solenoid valve which switches a vacuum line off, thereby allowing a vapor sample to emit from the end of a nozzle. Typically, this loop is 5-30 frames in duration with a 20 frame duration most common.

- 10 Loop 3. This is a sensor recovery loop. Another 5 volt trigger pulse is sent to a solenoid which switches back to its initial position, causing the vapor delivery system 290 to resume collection of the solvent vapor and carry it off to waste. Typically, this loop is of 30 frames duration in a 60 frame measurement and 20 frames duration in a 40 frame measurement.

- 15 For each loop, the frame rate and number of frames captured are adjusted to capture an appropriate number of data points for a sensor-analyte interaction. Loop duration and measurement times can be adjusted for each measurement to accommodate the response time of sensor elements, the duration of the analyte-sensor array interaction and the rise and decay of sensor responses to an analyte exposure.

#### B. Data Analysis

- 20 In the data analysis portion, pre-selected segments taken from a previously collected "focus" image are transferred to the sequence of images collected. These segments, drawn by the user, allow the mean pixel intensity to be measured in particular regions throughout the image field. Typically, they are drawn over individual pixels or groups of pixels of a fiber optic sensor array, each of which contains a discrete sensor or portion of a gradient sensor. The script then enters a loop that steps through each frame, measuring the mean pixel intensity within each segment, and places the values in data columns. The
- 25 resulting columns can then be plotted to yield the temporal response of each sensor element of interest. Before plotting, however, responses are typically background-subtracted and then "standardized". In one embodiment, the initial background signal, prior to exposing the sensor to an analyte, is subtracted from all data points and then normalized by dividing each data point value by the maximum signal. In this embodiment, the maximum response has a value of 1.0. In an alternative embodiment, the data
- 30 points are "standardized" by dividing the data for each sensor response by the first point data point. In this embodiment, all responses are thus normalized to start at a value of 1.0 for the purpose of facilitating the graphical display of all the temporal responses. In an alternative procedure, the initial background signal, prior to exposing the sensor to an analyte, is adjusted to start at a value of 0.0 by subtracting the integer 1.0 from the standardized data points.



Conventional statistical analysis of sensor temporal responses may be employed where it is desirable to compare the discriminating capacities of either discrete copolymer sensors or gradient copolymer sensors for applications as individual sensors or as sensor elements in cross-reactive sensor arrays. Cluster analysis methods which employ graphical representations of statistically determined dissimilarities between sensors are particularly useful in comparing sensor discriminating capabilities. Such statistical analytical methods are conventionally known and applied in the chemical arts and detailed discussion of these methods are provided by both R.G. Brereton [see *Chemometrics: Applications of Mathematics and Statistics to Laboratory Systems*, Ellis Horwood (New York, 1990), p. 244-266] and D. Livingstone [see *Data Analysis for Chemists*, Oxford Univ. Press (New York, 1995), p. 38-40, 81-92, 103-112, 170-174].

Cluster analysis methods may be employed for quantitative comparison of discrete sensor responses to specific analytes by characterizing the "dissimilarity" between the temporal responses of each copolymer sensor type to an analyte. While a variety of statistical measures are available for quantifying sensor dissimilarity, a particularly useful approach involves representing each sensor response as a point in multidimensional space and then calculating the Euclidean distance between points [see T.A. Dickinson, et al., *Anal. Chem.* 69(17):3413(Sept. 1. 1997). In this method, the equation

$$d_{ij} = \left[ \sum_1^T (f_{i,t} - f_{j,t})^2 \right]^{1/2}$$

defines the statistical distance between the temporal responses of sensors *i* and *j*. The squared difference between the fluorescence signal from the two sensors at each time point,  $f_{i,t}$  and  $f_{j,t}$ , is summed over all time points of the sensor response measurements. A diagonally symmetrical distance matrix is generated for the entire sensor array and the distance sare used to construct a dendrogram allowing the identification of groups or clusters having similar responses. A sample application of this method is provided in Example 6 for analysis of responses of discrete combinatorial copolymer sensors to benzene.

As an optional approach for quantifying dissimilarities of gradient sensor regions of interest to specific analytes, a matrix of pairwise Euclidean distances may be generated as above for each ROI of a gradient sensor and a control and the distance values for each matrix are totaled to provide a comparison of an average summed distance for the gradient and control sensor regions of interest. A conventional multidimensional scaling plot may be utilized for graphical display of observed dissimilarity between the gradient and control sensors. This graphical method provides a two dimensional representation of the pairwise dissimilarities between responses of regions of interest for each sensor type. A sample application of this method is provided in Example 7 for analysis of responses of gradient combinatorial copolymer sensors to benzene.

In one preferred embodiment, the reduced sensor array data are employed in a neural network analysis for identifying analytes according to the method disclosed in White, et al., *Anal. Chem.* 68:2193-2202 (1996).

#### 5. Sensor Element Signal Enhancement

- 5 In one alternative embodiment, to improve array detection response and sensitivity, the optical response signals from a large number of discrete sensor elements within each sensor type may be summed by simply adding the baseline-adjusted intensity values of all responses at each time point, generating a new temporal response comprised of the sum of all sensor responses for each sensor type. Signal summing can be performed in real time or during post-data acquisition data reduction and analysis. In
- 10 one embodiment, signal summing is performed with Excel (Microsoft, Redmond, WA), a commercial spreadsheet program, after optical response data are collected.

- In a typical procedure, the standardized optical responses are adjusted to start at a value of 0.0 by subtracting the integer 1.0 from all normalized data points. Doing this allows the baseline-loop data to remain at zero even when summed together and the random response signal noise is canceled out.
- 15 The vapor pulse-loop temporal region, however, exhibits a characteristic change in response, either positive, negative or neutral, prior to the vapor pulse and often requires a baseline adjustment to overcome noise associated with drift in the first few data points due to charge buildup in the CCD camera. If no drift is present, typically the baseline from the first data point for each sensor element is subtracted from all the response data for the same sensor element. If drift is observed, the average
- 20 baseline from the first ten data points for each sensor element sensor is subtracted from all the response data for the same sensor element.

- By applying this baseline adjustment, when multiple sensor element type responses are added together they can be amplified while the baseline remains at zero. Since all sensor elements respond at the same time to the vapor pulse, they all see the pulse at the exact same time and there is no registering
- 25 or adjusting needed for overlaying their responses. Cumulative response data are generated by simply adding all data points in successive time intervals. This final column, comprised of the sum of all data points at a particular time interval, may then be compared or plotted with the individual sensor responses to determine the extent of signal enhancement or improved signal-to-noise ratios.

#### EXAMPLE 1

- 30 A copolymer sensor array comprising discrete sensors formed by the combinatorial method of the present invention was made with two prepolymers, which were partially polymerized, and four discrete prepolymer composition ratios. An (80-85%) dimethyl-(1520%) (acryloxypropyl)methylsiloxane copolymer, PS802, available from Gelest Inc. (Tullytown, PA) and methyl methacrylate, MMA, available from Aldrich (Milwaukee, WI) were selected as example monomers for copolymerization. In one
- 35 experiment, a combinatorial sensor array was prepared from separate prepolymer solutions containing

PS802/MMA combinations ranging from 0.0, 5.0, 20.0 and 50.0% MMA in PS802. In an alternative experiment, a second combinatorial sensor array was prepared from separate prepolymer solutions containing PS802/MMA combinations ranging from 0.0, 6.7, 33.3, and 50.0% MMA in PS802. In preparing each sensor array, dye solution of 1mg/mL Nile Red in chloroform and a 30mg/mL solution of benzoin ethyl ether initiator in toluene was added to each prepolymer solution. For both arrays, the prepolymer mixture-dye-initiator solutions were individually polymerized onto the end of a coherent imaging fiber using the photodeposition system described herein, to form a plurality of discrete sensing regions across the face of the fiber. Each of the solutions was polymerized for 5 seconds, resulting in approximately 45µm-diameter polymer hemispheres or cones attached to the distal end of the fiber bundle. Duplicates of each monomer combination mixture were polymerized, yielding a total of eight spatially separated sensor elements. A fluorescence image of the first sensor array comprising sensors containing 0.00, 5.0, 20.0 and 50.0% MMA in PS802 is shown in the PS802/MMA sensor array in Fig. 3. Replicate sensor elements were deposited for each of the prepolymer combination mixtures.

The second sensor array comprising sensors containing 0.00, 6.7, 33.3 and 50.0% MMA in PS802 was tested by exposing the array to a variety of analytes. Figs. 4a-d depict the fluorescence output of this sensor array upon exposure to pulses of various saturated vapors. As shown in these figures, each of the four prepolymer combinations produces a unique temporal optical response for each of the four analytes tested: benzene (A), hexane (B), 2-propanol (C) and ethyl acetate (D). These results indicate that the optical responses for each copolymer sensor having a discrete prepolymer combination produce a unique and distinguishable response to the same pulse of four different analytes and thus provide a characteristic discriminating measure of the presence of a variety of target analytes. Figs. 4a-d also indicate that the sensor response for each copolymer sensor type is reproducible and that replicates for each prepolymer combination produce similar response curves.

The diversity in copolymer sensor responses exhibited by the various copolymer sensor types shown in Figs. 4a-d has utility for detecting and discriminating a variety of target analytes, demonstrating positive, negative, and biphasic fluorescence changes in response to each analyte tested. The individual optical response characteristics for each of the copolymer sensor types contains numerous additional distinguishing features which are useful for discriminating analytes as well, including different rise times, slopes, recovery rates, and sharp peaks associated with each sensor's characteristic response to each of the analytes tested. As shown by the results of Figs. 4a-d, this discrimination capacity is seen both between classes of analytes, such as aromatic and alcohol compounds, as well as within a class of analytes, such as methanol and propanol.

From the results shown in Figs. 4a-d, it is important to note that there does not appear to be any obvious progression or linear relationship between the optical response characteristics of the prepolymer combinations and the prepolymer composition ratios for each copolymer sensor type. The response characteristics of the combinatorial copolymer sensors are thus not simply related to the proportion of

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the two oligomers or monomers utilized. The differences observed in the optical response characteristics of these copolymer sensors is due to a difference in the polarity, swelling characteristics, or chemical absorption of the copolymerized matrices formed from each prepolymer combination. These results demonstrate that the combinatorial polymer sensors of the present invention provide

5 unique characteristic optical responses from each monomer combination, that such sensors exhibit a high degree of diversity in optical response to a variety of target analytes, that such sensors can discriminate between classes and within classes of target analyte compounds, that the sensor response is reproducible, and that the response characteristics are not simply related to proportional ratios of the oligomer or monomer combinations.

10

## EXAMPLE 2

A copolymer gradient sensor was prepared by photodepositing a polymer strip from a prepolymer mixture having a time-varying oligomer or monomer composition. The distal end of a fiber optic bundle was immersed in 1 ml of a stirred solution containing PS901.5 (acryloxypropylmethyl siloxane), from United Chemical Technologies Inc. (Bristol, PA), in chloroform (1:1), with 27mg/mL of benzoin ethyl

15 ether initiator. For photodeposition of the copolymer, a UV light beam was focussed through a pinhole aperture and scanned across the face of the fiber optic bundle at a rate of 25µm/s while 1 ml of PS802 prepolymer solution (2:3 in chloroform, 30mg/mL BEE) was injected into the PS901.5 mixture using an Orion Research (Boston, Ma) Model M361 syringe pump. Both prepolymer solutions were flushed with nitrogen for 20 minutes prior to mixing and polymerization. The beam scanning and mixing of the two

20 prepolymer solutions occurred simultaneously and continuously over a twenty-two second photopolymerization period. This method of photodeposition produced a copolymerized polymer strip with a continuously varying structure and composition gradient due to the photodeposition of a prepolymer solution ranging from pure PS901.5 to a 5:4 mixture of PS901.5 and PS802. Using the same procedure, but without the addition of a second monomer to the reaction vessel, a

25 single-component polymer control strip of PS901.5 was polymerized onto the face of the same fiber optic bundle. Both of the photodeposited polymer strips were soaked in a Nile Red (1mg/mL in toluene) solution for 30 minutes, rinsed with ethanol, and allowed to dry overnight.

For the resultant PS901.5/PS802 polymerized copolymer gradient sensor (Fig. 5a), twelve regions of interest (ROI's) were drawn at various locations along the vertical axes of both the copolymer gradient

30 sensor and an adjacent, single-component PS901.5 polymer control sensor stripe. The temporal fluorescence changes for each of the twelve ROI's of the copolymer sensor in response to vapor pulses of benzene (Fig. 6a) and methanol (Fig. 7a) were found to cover a wide range of shapes and intensities. The corresponding ROI's of the PS901.5 polymer control sensor (Fig. 5b), however, yielded twelve temporal responses of roughly the same shape in response to benzene (Fig. 6b) or methanol (Fig. 7b).

35

## EXAMPLE 3

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A copolymer gradient sensor array was also prepared using a PS802/MMA monomer combination, according to the method described in Example 2. For this copolymer gradient sensor, a 1:1 solution of MMA monomer in chloroform was slowly added to a 1:1 prepolymer solution of PS802 in chloroform, each prepolymer solution containing 30 mg/ml of benzoin ethyl ether in toluene. During the continuous and simultaneous addition and mixing of the MMA solution with the PS802 solution, a UV light beam was focussed through a pinhole aperture and scanned across the face of a fiber optic bundle which was immersed in the solution mixture, yielding a continuously varying, copolymer structure and composition gradient across the fiber bundle end face. This method of photodeposition produced a copolymerized polymer strip with a continuously varying structure and composition gradient due the photodeposition of a prepolymer solution ranging from pure PS802 to a 1:1 mixture of PS802 and MMA.

For the resultant PS802/MMA polymerized copolymer gradient sensor (Fig. 8a), twelve regions of interest (ROI's) were drawn at various locations along the vertical axes of both the copolymer gradient sensor and an adjacent, single-component PS802 polymer sensor control strip. The temporal fluorescence changes for each of the twelve ROI's of the copolymer gradient sensor in response to vapor pulses of hexane (Fig. 9a), methanol (Fig. 10a) and benzene (Fig. 11a) provide a broad diversity in optical response which is useful for discriminating a variety of target analytes. The corresponding ROI's of the PS802 polymer sensor control strip (Fig. 8b), however, yielded twelve temporal responses of roughly the same shape in response to the analytes hexane (Fig. 9b), methanol (Fig. 10b), or benzene (Fig. 11b). Thus, direct comparison of the observed optical responses for the copolymer gradient sensor and the polymer sensor control strip to the various analytes demonstrates the superior diversity in response and analyte discriminating capability of the copolymer gradient sensors and sensor arrays of the present invention.

#### EXAMPLE 4

The cluster analysis method was used to compare the dissimilarity of responses of the combinatorial copolymer sensors of Example 1 to benzene. A dendrogram was constructed for identifying and isolating sensors having similar responses. The results are shown in the dendrogram of Fig. 12 where clusters of responses for similar copolymer sensor composition are shown for 0% MMA (1 and 2), 6.7% MMA (3 and 4), 33.3% MMA (5 and 6) and 50% MMA (7 and 8) and dissimilarity of responses between cluster groups are shown for the different copolymer sensor compositions. Fig. 12 also demonstrates the non-linear relationship between copolymer composition and sensor response where the responses of sensors with a 50% MMA composition are more closely related to the responses of sensors with 0% and 6.7% MMA compositions than the 33.3% MMA composition.

#### EXAMPLE 5

The cluster analysis method was used to compare the dissimilarity of responses of the combinatorial copolymer gradient sensor and control sensor of Example 2 to benzene. The responses of twelve regions of interest for each sensor type were compared in a multidimensional scaling plot for

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representing the increased dissimilarity between the gradient sensor and control sensor regions of interest. The results are shown in the multi-dimensional scaling plot of Fig. 13 where pairwise dissimilarities between regions of interest for each sensor type are plotted. As demonstrated by this plot, the gradient sensor responses are more widely scattered than the control sensor, indicating greater diversity in sensor response.

5

Having described the preferred embodiments of the invention, it will now become apparent to one of skill in the art that other embodiments incorporating the concepts may be used. Therefore, it is not intended to limit the invention to the disclosed embodiments but rather the invention should be limited only by the spirit and scope of the following claims.

What is claimed is:

1. A sensor array comprising:
  - a) a substrate comprising discrete sites; and
  - b) a plurality of copolymer sensors distributed on said sites, each of said sensors comprising  
5 a discrete mixture of at least a first and a second polymerizable material.
2. A sensor array according to claim 1 wherein said copolymer sensors are on microspheres distributed on said sites.
3. A sensor array according to claim 1 or 2 wherein said substrate is a fiber optic array.
4. A sensor array according to claim 1, 2 or 3 wherein said sensors further comprise at least one dye.
- 10 5. A sensor array according to claim 1, 2, 3 or 4 further comprising a detector for detecting sensor responses.
6. A sensor array according to claim 5 wherein said detector is an optical detector.
7. A method of detecting the presence of a target analyte in a sample comprising:
  - a) adding said sample to a sensor array comprising:
    - 15 i) a substrate comprising discrete sites; and
    - ii) a plurality of copolymer sensors distributed on said sites, each of said sensors comprising a discrete mixture of at least a first and a second polymerizable material;
  - b) detecting an alteration in a property selected from a group consisting of mass, temperature, heat, light, voltage, current, polarity, intensity, refractive index, polarization, phase, wavelength,  
20 frequency, periodicity, and dimension.
8. A method according to claim 7 wherein said copolymer sensors are on microspheres distributed on said sites.
9. A method according to claim 7 or 8 wherein said substrate is a fiber optic array.
10. A method according to claim 7, 8 or 9 wherein said sensors further comprise at least one dye.
- 25 11. A method according to claim 7, 8, 9 or 10 further comprising a detector for detecting sensor responses.
12. A method according to claim 11 wherein said detector is an optical detector.

13. A method of making a sensor array comprising:
- a) polymerizing in a plurality of predetermined ratios:
    - i) a first solution comprising a first polymerizable material; and
    - ii) a second solution comprising a second polymerizable material;
- 5       said ratios being unique for at least two of said solutions, such that each polymerization reaction results in a different copolymer sensor; and
- b) distributing said copolymer sensors on a substrate.
14. A method according to claim 13 wherein said substrate comprises a fiber optic array.
15. A method according to claim 13 wherein said copolymer sensors are polymerized onto
- 10       microspheres that are distributed on said substrate.
16. A method according to claim 13 wherein said copolymer sensors are polymerized into microspheres that are distributed on said substrate.



## AMENDED CLAIMS

[received by the International Bureau on 5 May 2000 (05.05.00);  
original claims 1-16 replaced by new claims 1-16 (2 pages)]

1. A combinatorial polymer sensor comprising:
  - a) a substrate; and
  - b) a continuous gradient of copolymers distributed on said substrate, said gradient
- 5 comprising a continuously varying mixture of at least a first and a second polymerizable material.
2. A sensor array according to claim 1 wherein said substrate is a fiber optic array.
3. A sensor array according to claim 1 or 2 wherein said sensors further comprise at least one dye.
4. A sensor array according to claim 1, 2 or 3 further comprising a detector for detecting sensor
- 10 responses.
5. A sensor array according to claim 4 wherein said detector is an optical detector.
6. A method of detecting the presence of a target analyte in a sample comprising:
  - a) adding said sample to a sensor array comprising:
    - i) a substrate; and
    - 15 ii) a continuous gradient of copolymers distributed on said substrate, said gradient comprising a continuously varying mixture of at least a first and a second polymerizable material;
  - b) detecting an alteration in a property selected from a group consisting of mass,
  - 20 temperature, heat, light, voltage, current, polarity, intensity, refractive index, polarization, phase, wavelength, frequency, periodicity, and dimension.
7. A method according to claim 6 wherein said substrate is a fiber optic array.
8. A method according to claim 6 or 7, wherein said sensors further comprise at least one dye.
9. A method according to claim 6, 7 or 8, further comprising a detector for detecting sensor responses.
- 25 10. A method according to claim 9 wherein said detector is an optical detector.
11. A method of making a sensor array comprising:
  - a) polymerizing in a plurality of predetermined ratios:
    - i) a first solution comprising a first polymerizable material; and

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- 5                   ii) a second solution comprising a second polymerizable material;  
said ratios being unique for at least two of said solutions, such that each polymerization  
reaction results in a different copolymer sensor; and  
b) distributing said copolymer sensors on a substrate, said substrate comprising a surface  
comprising discrete sites.

12. A method according to claim 11 wherein said copolymer sensors are polymerized onto  
microspheres that are distributed on said substrate.

13. A method according to claim 11 wherein said copolymer sensors are polymerized into  
microspheres that are distributed on said substrate.

- 10           14. A method of making a combinatorial sensor comprising:

a) providing:

i) a substrate;

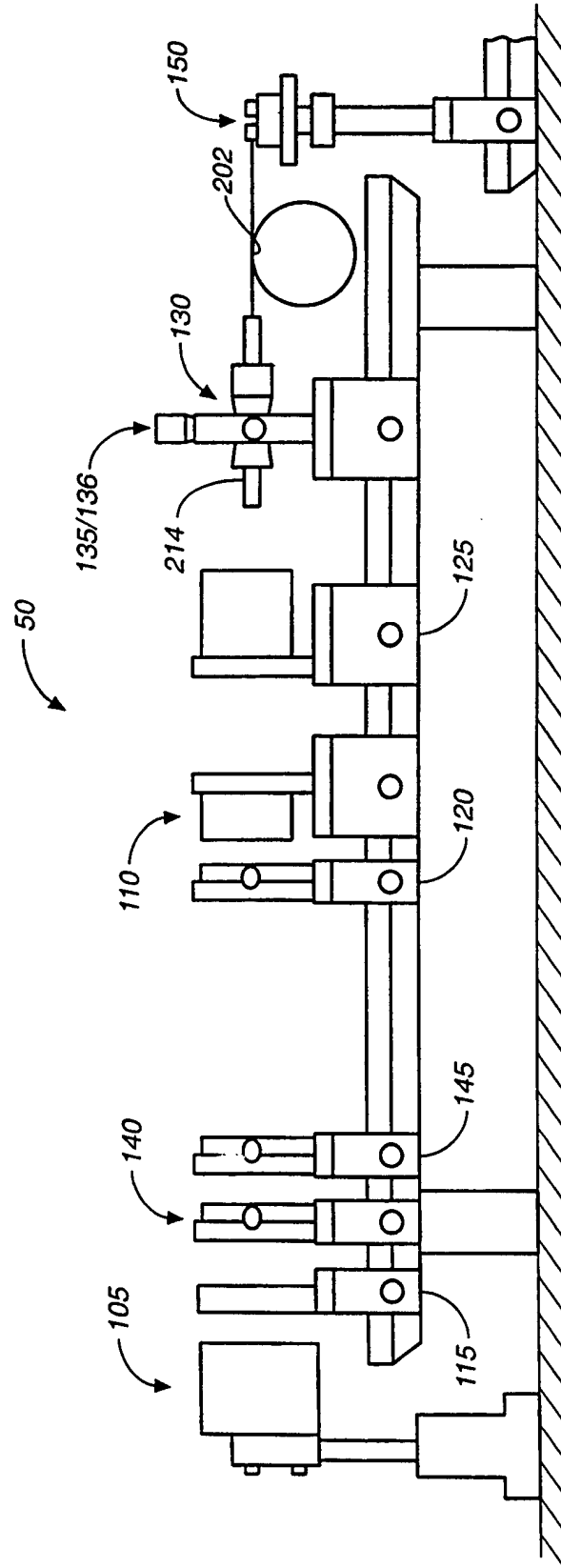
ii) a continuously varying mixture comprising at least a first and a second  
polymerizable material; and

- 15                   b) polymerizing on said substrate at a predetermined rate said continuously varying  
mixture.

15. A method according to claim 11 or 14, further comprising combining at least one dye to said  
continuously varying mixture.

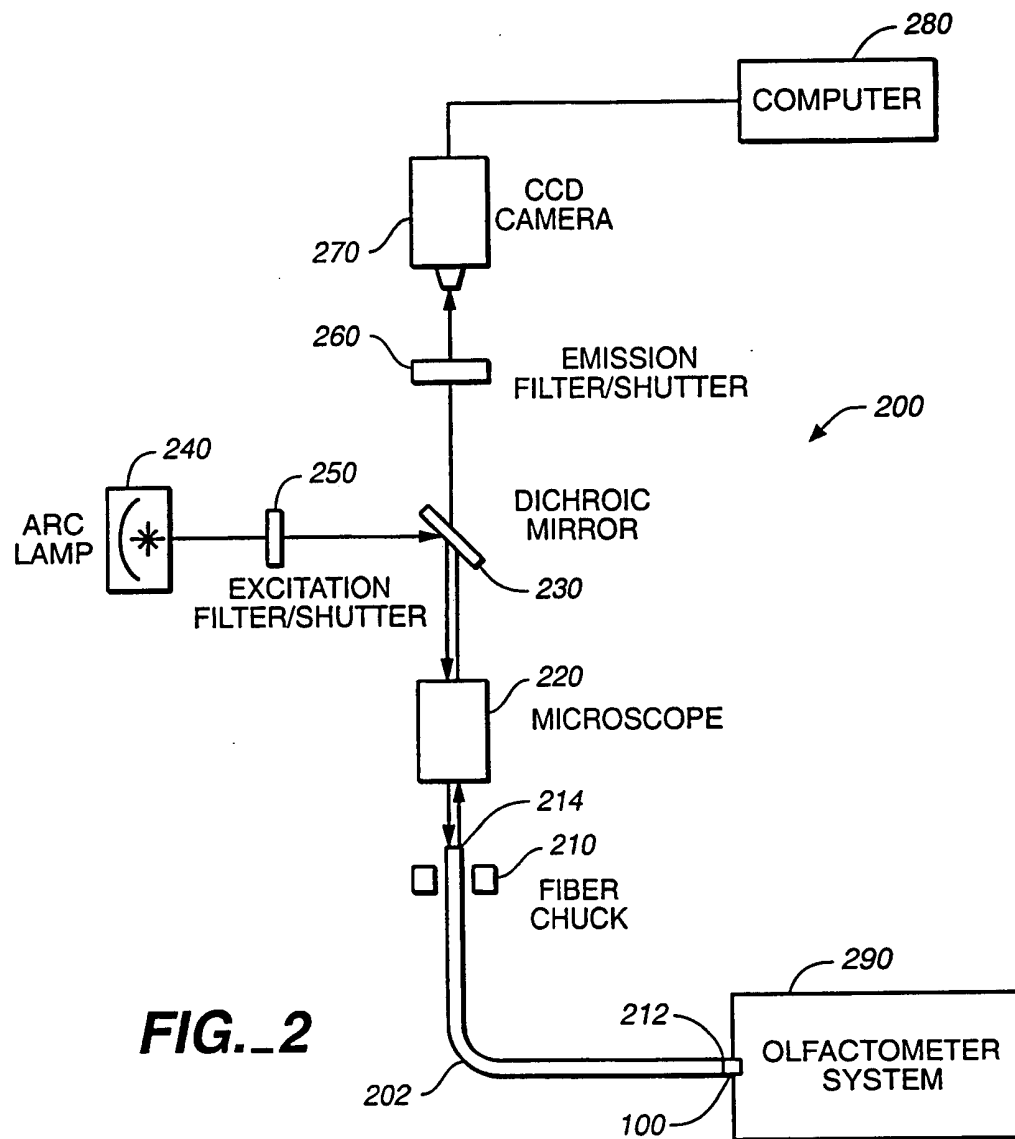
16. A method according to claim 11 or 14, wherein said substrate is a fiber optic array.

20

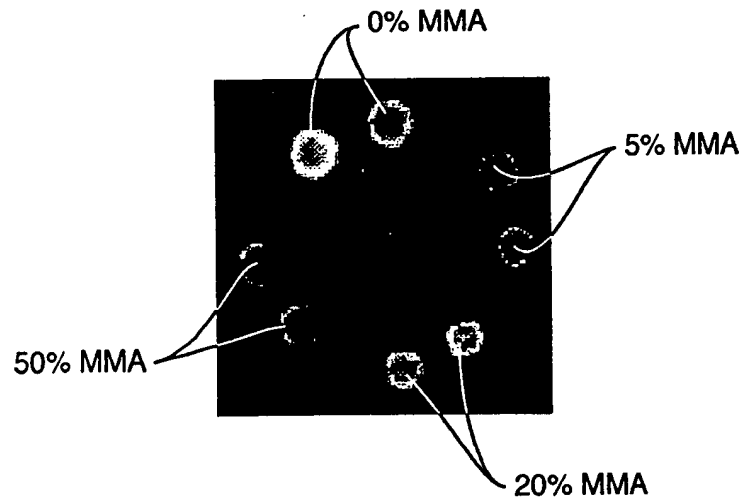


**FIG. 1**

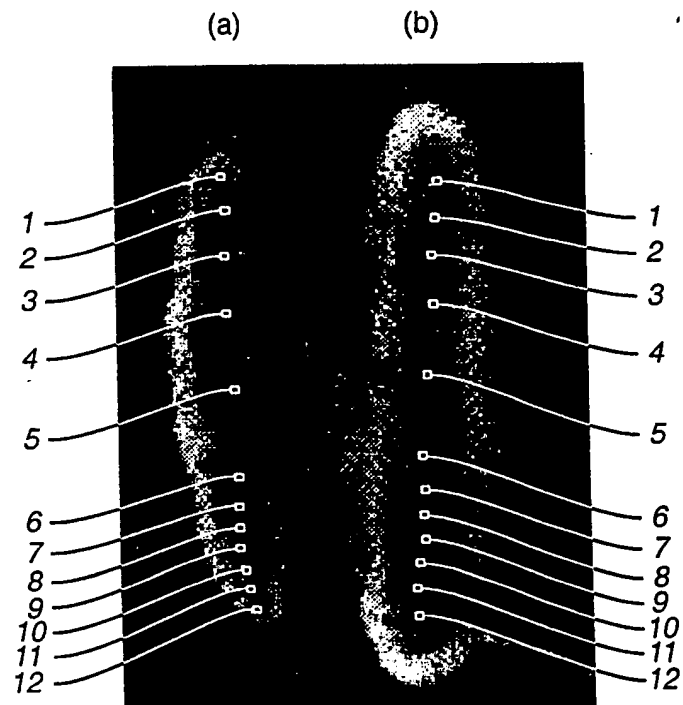
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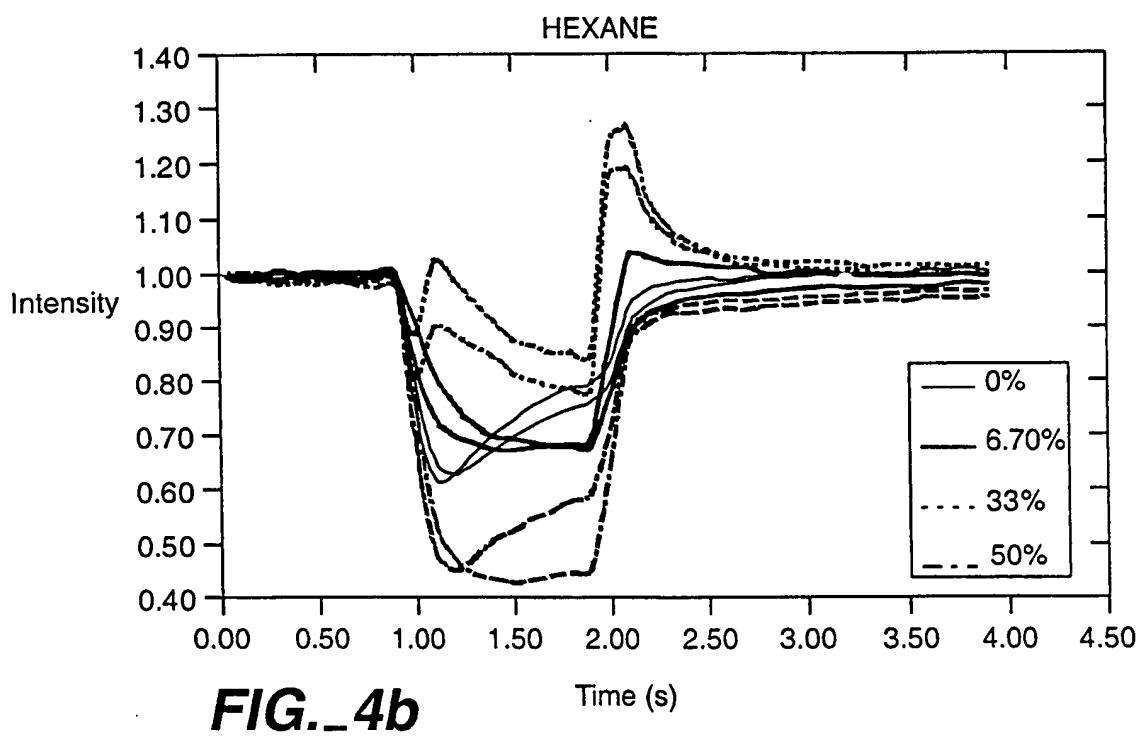
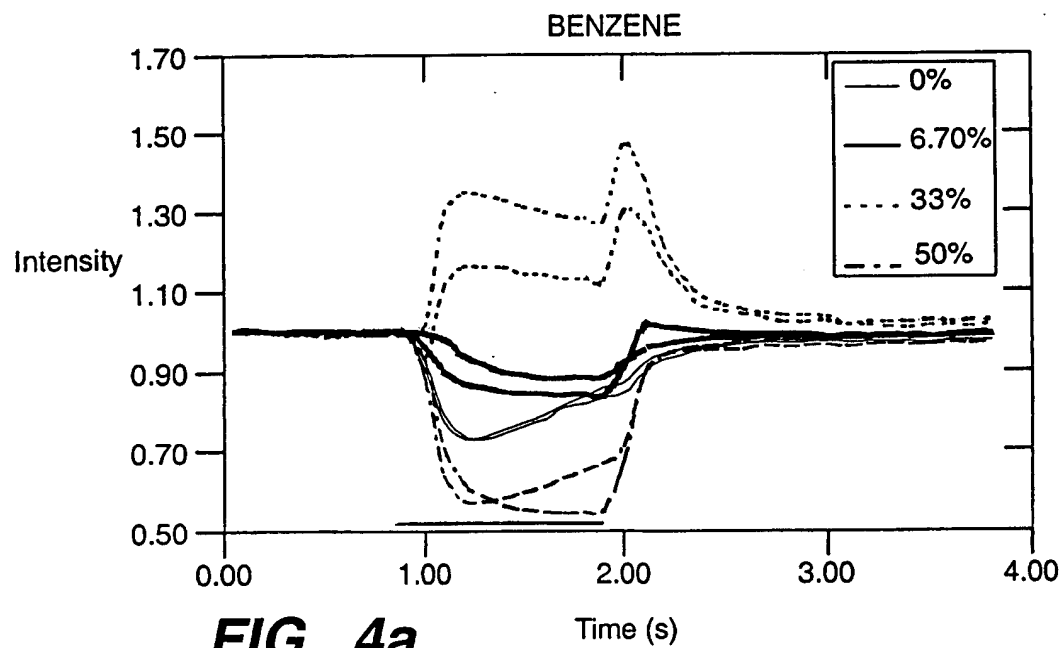
**FIG. 3**



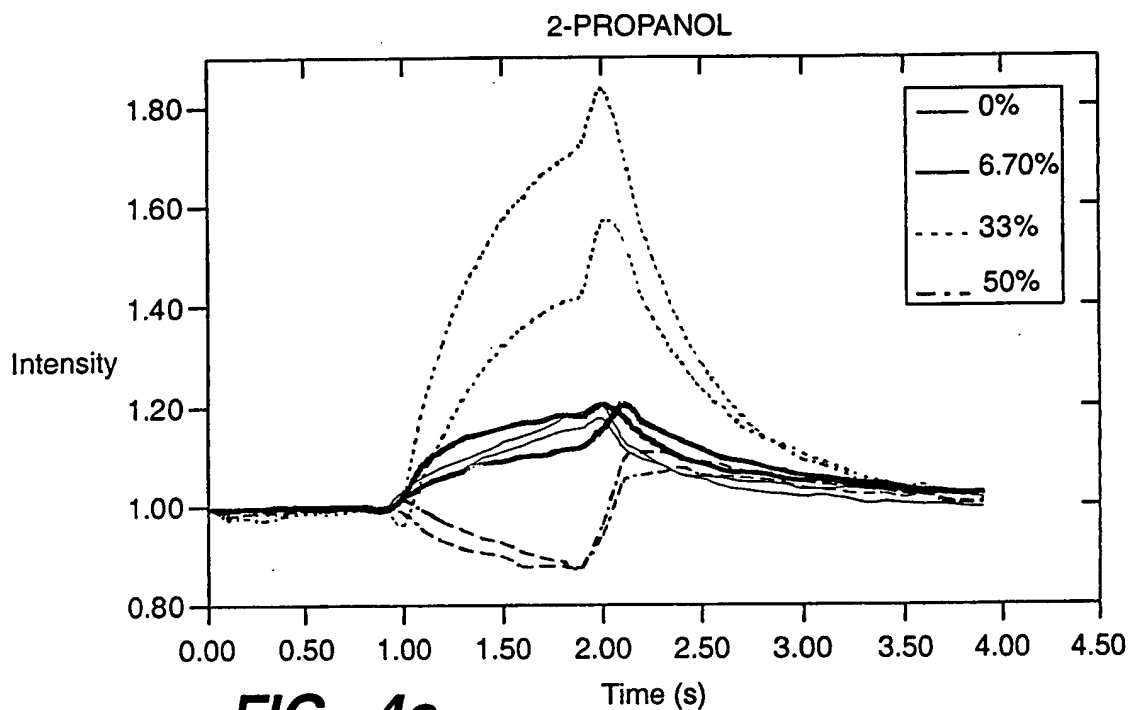
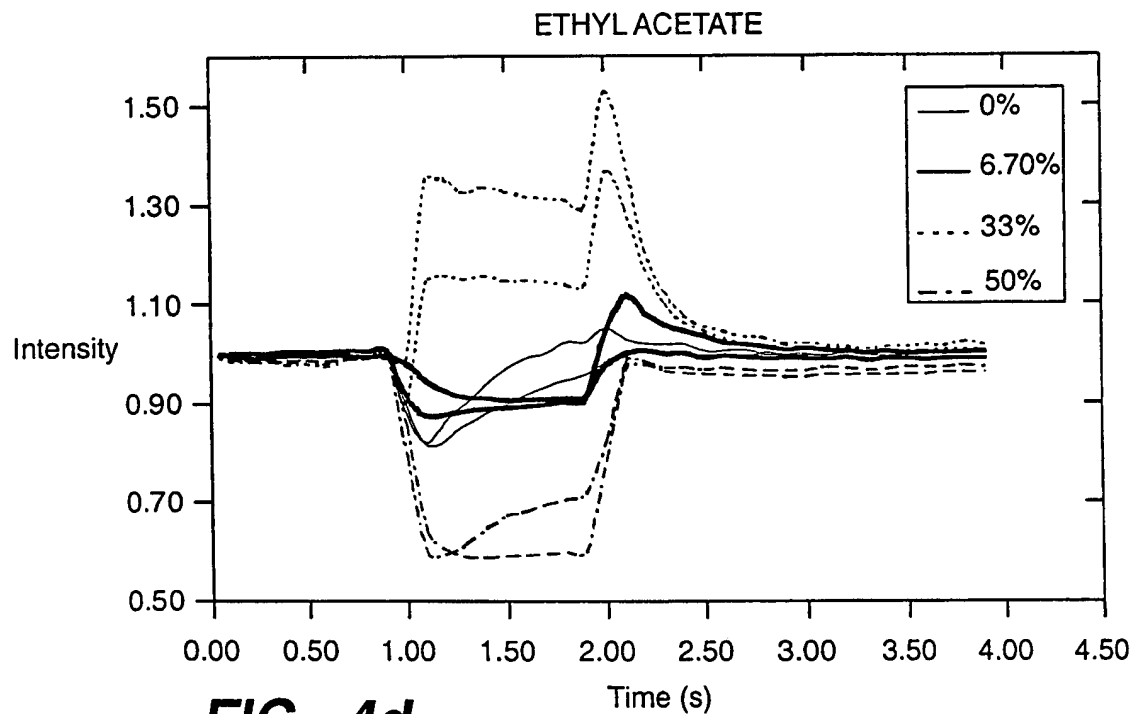
**FIG. 5a-b**

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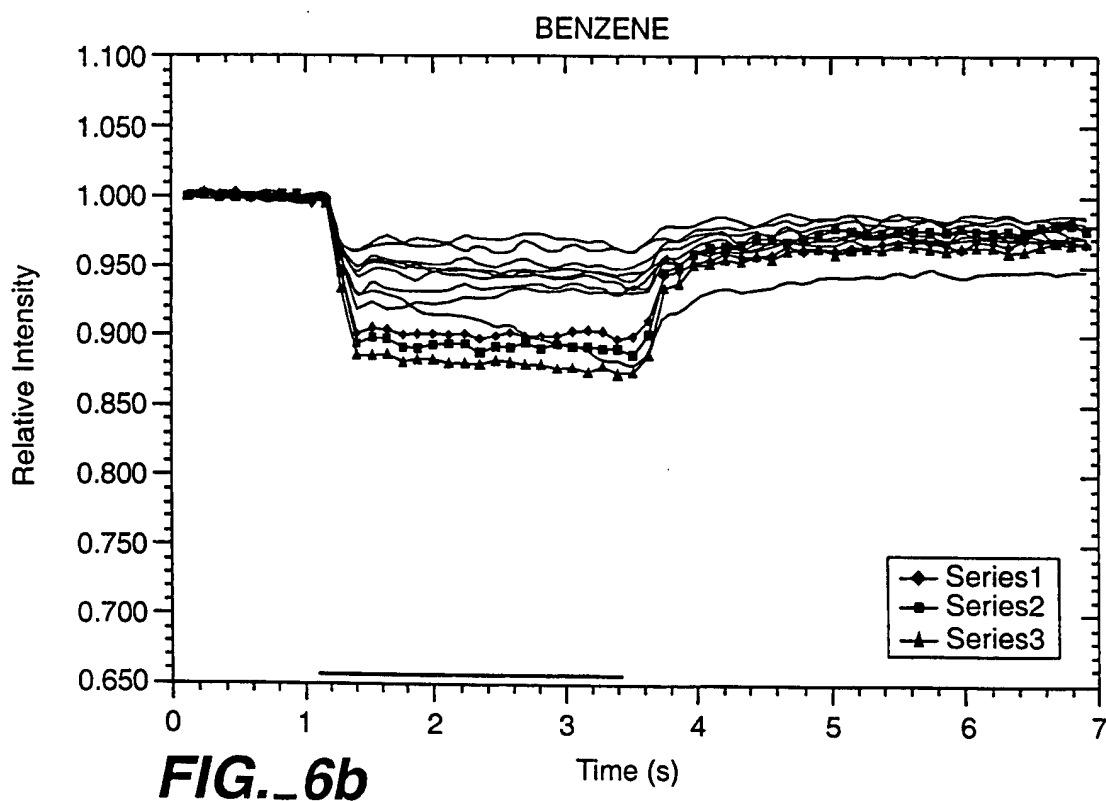
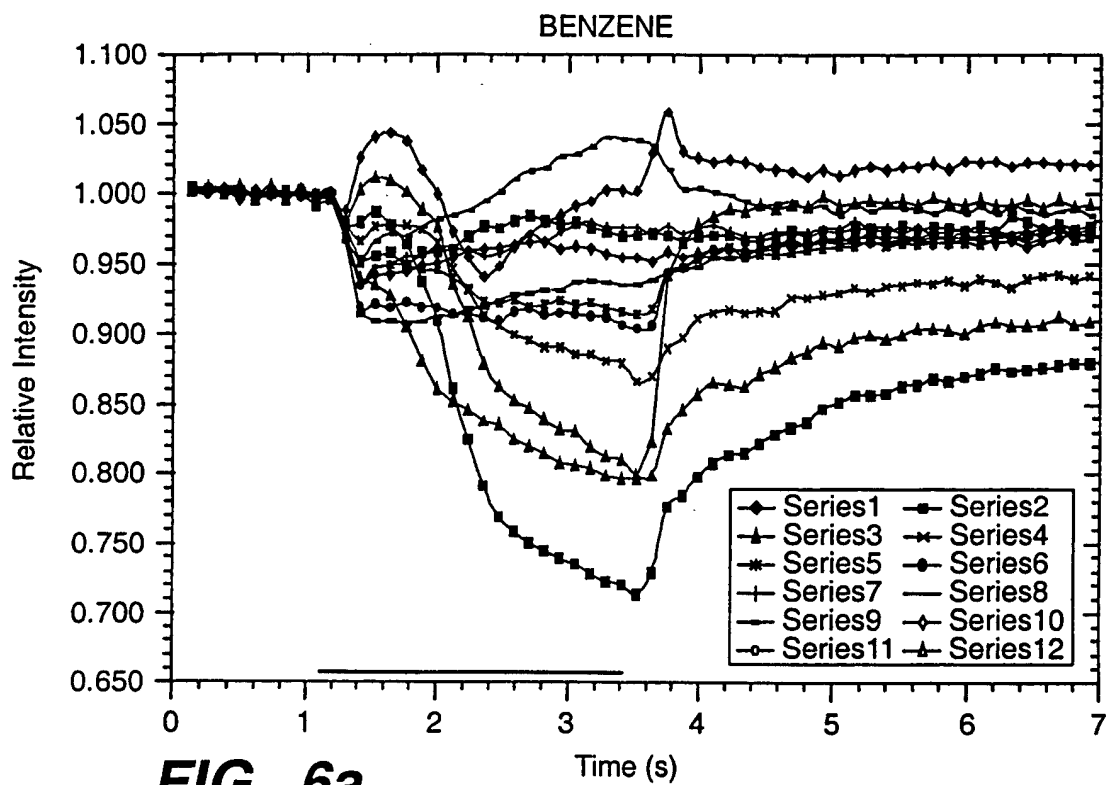
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**FIG.\_4c****FIG.\_4d**

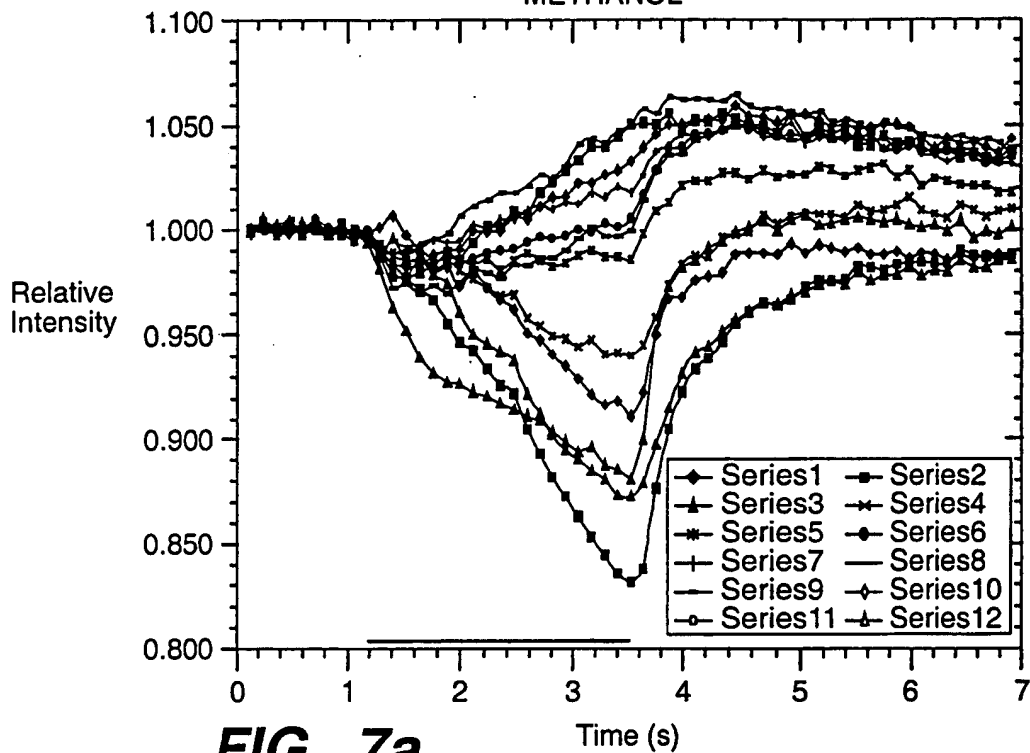
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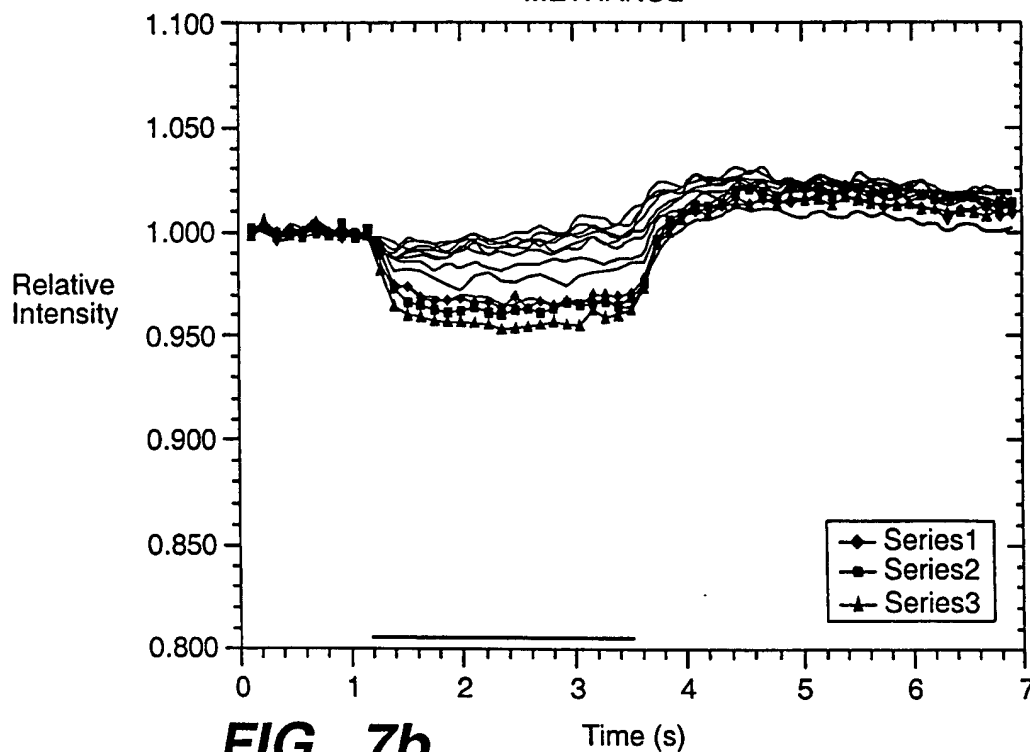


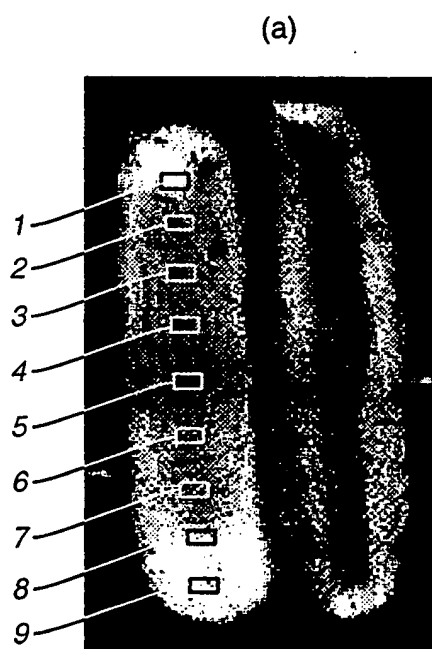
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METHANOL

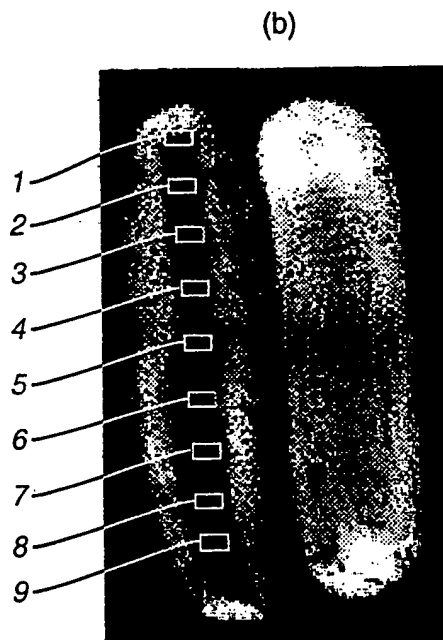


METHANOL



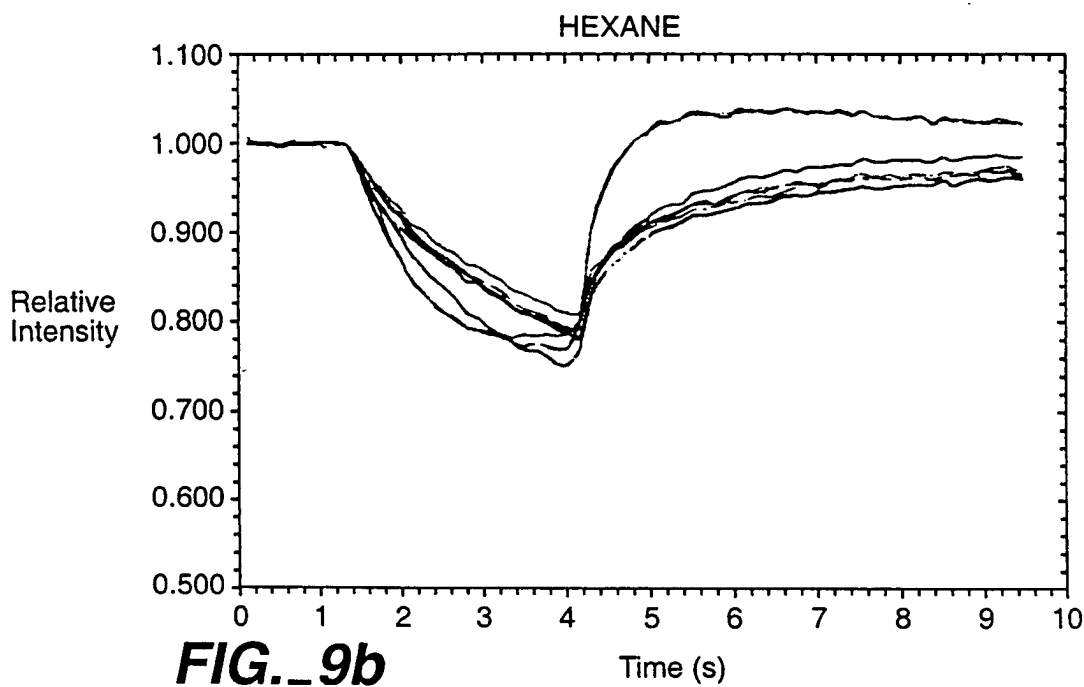
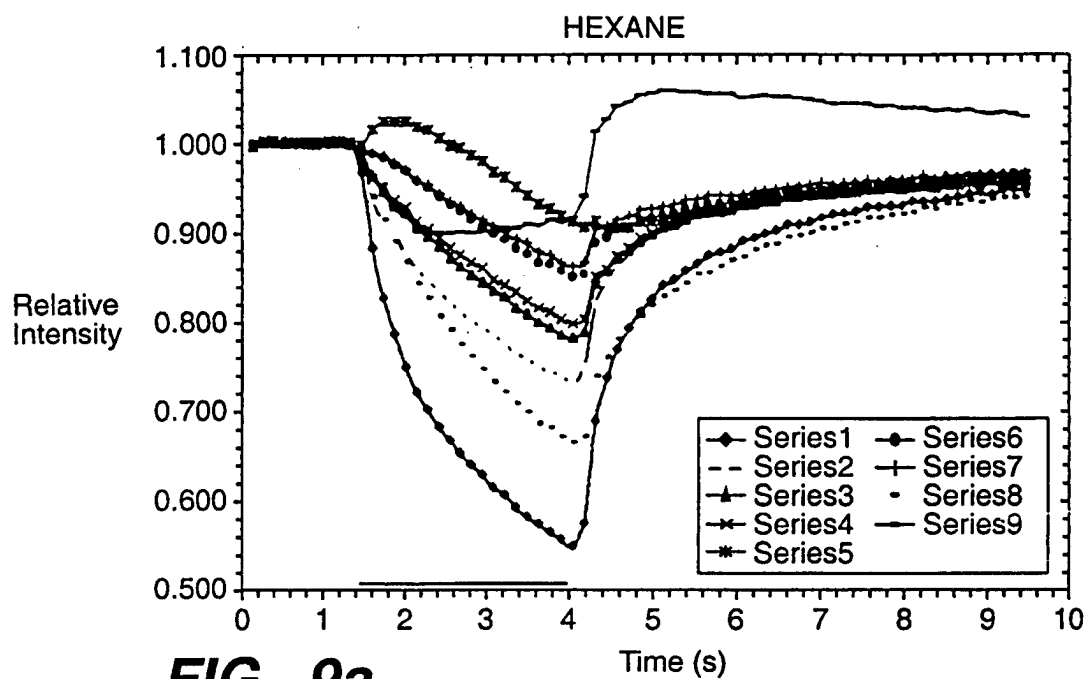


**FIG.\_8a**

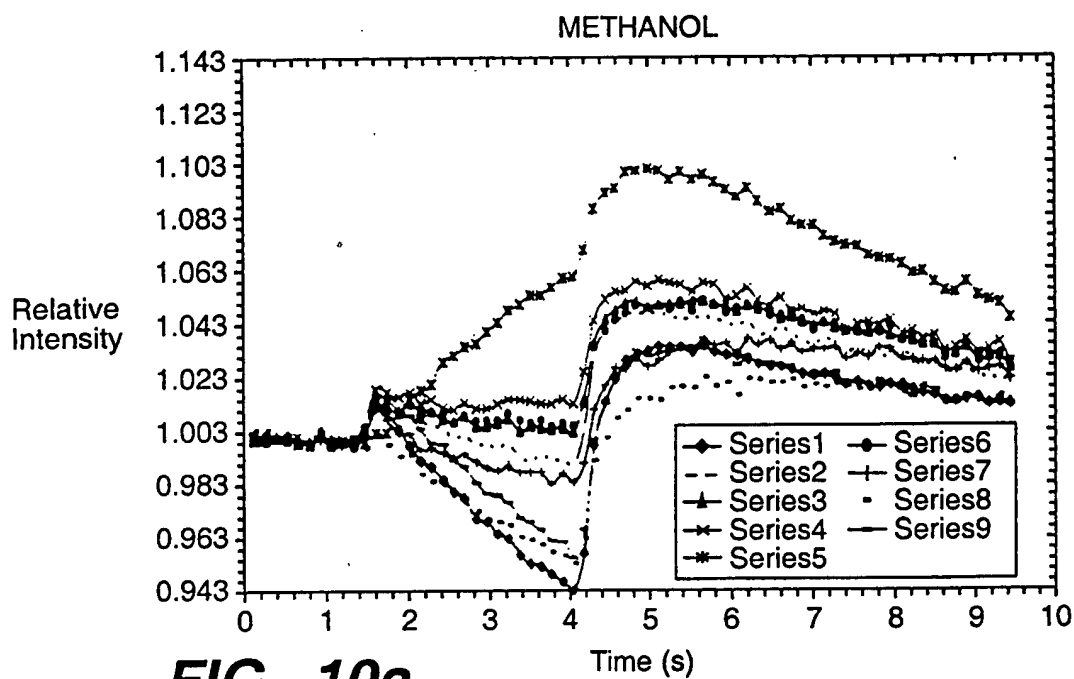
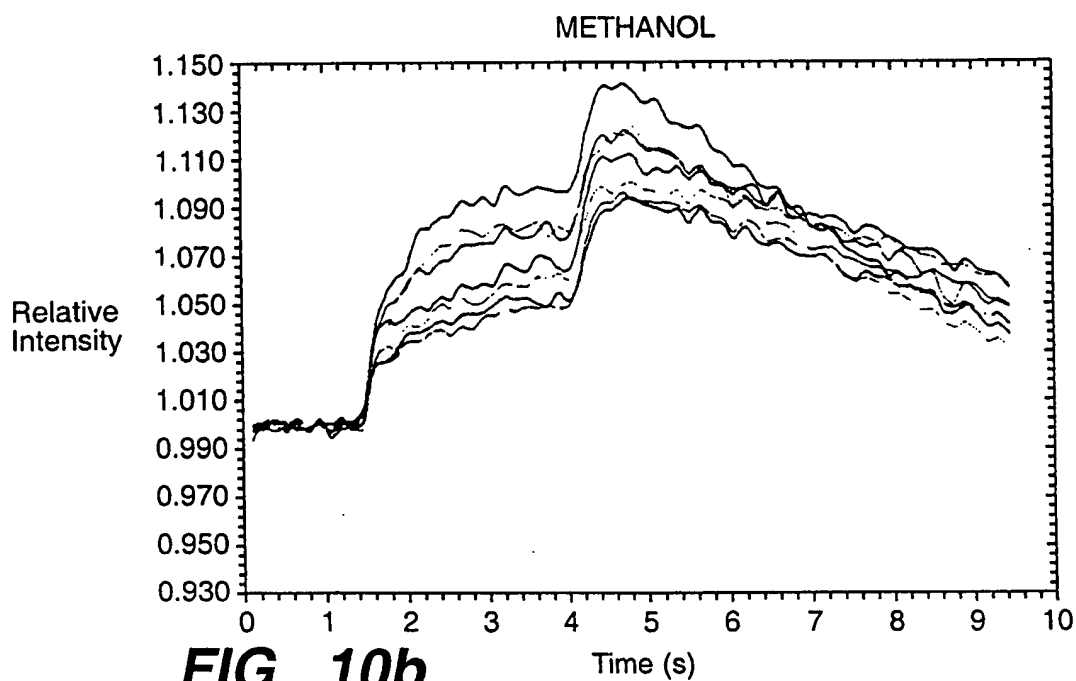


**FIG.\_8b**

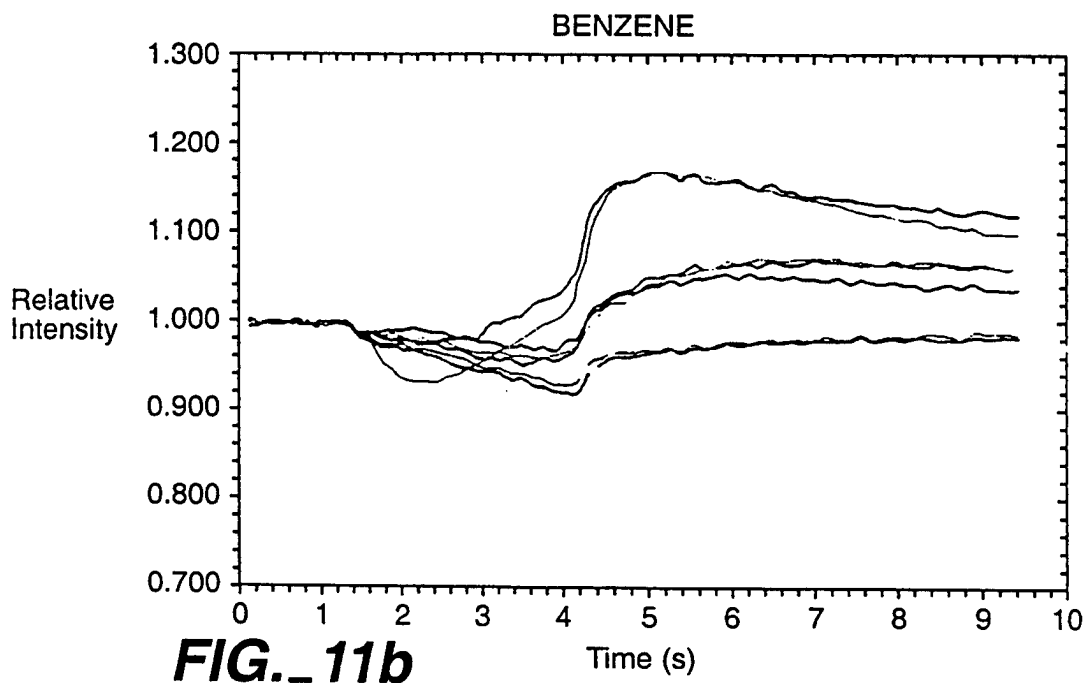
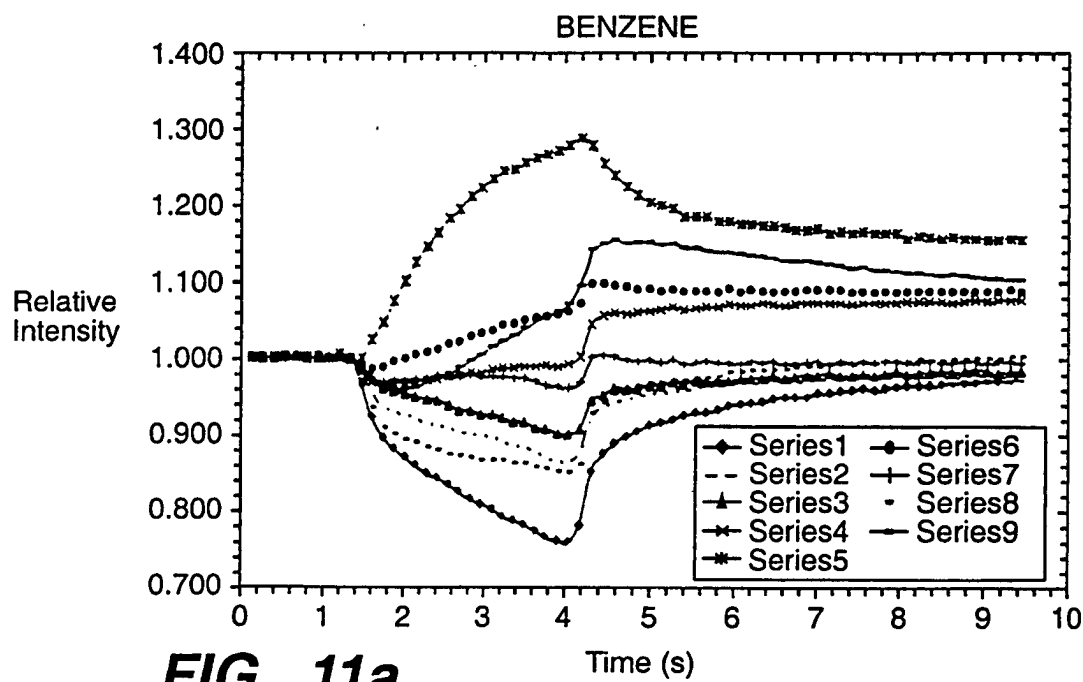
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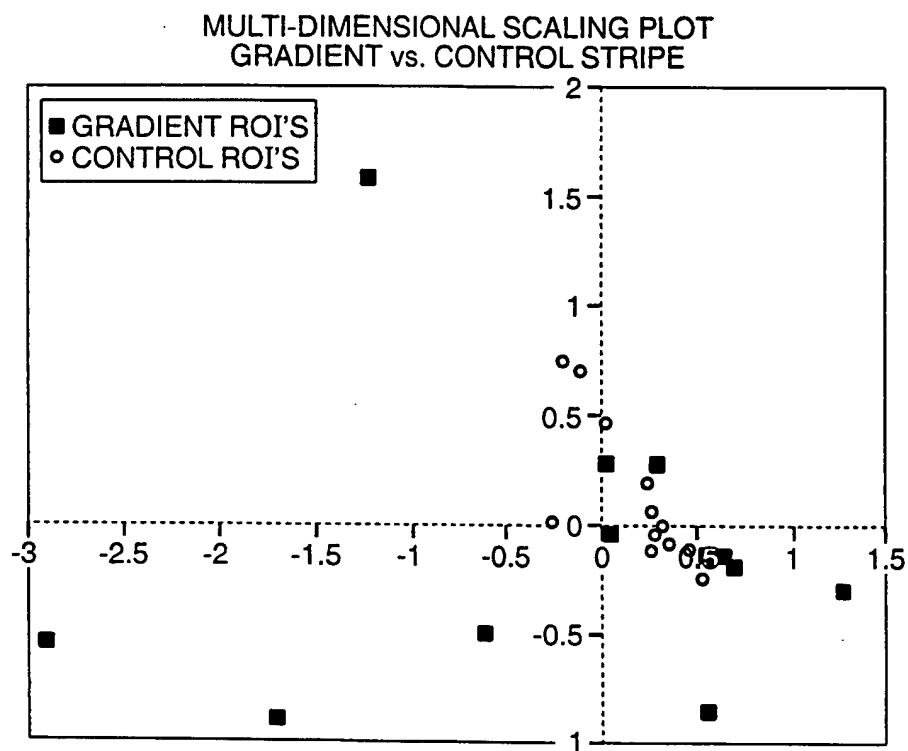
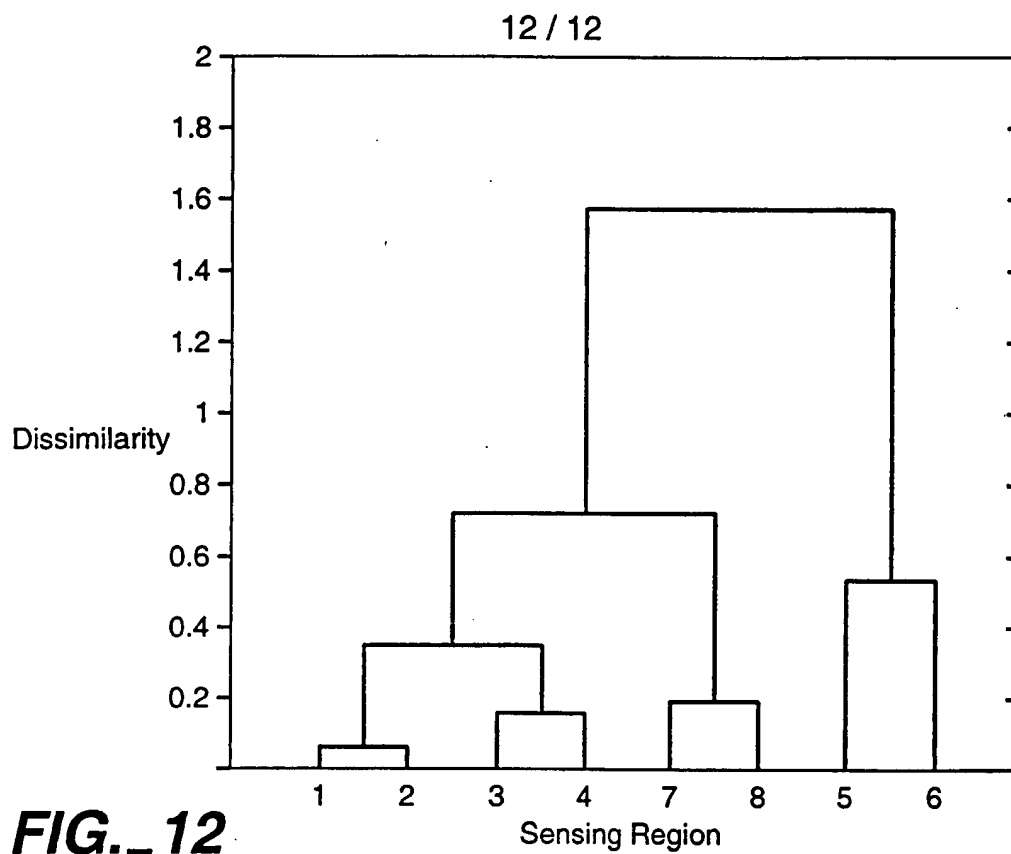


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**FIG.\_10a****FIG.\_10b**

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## INTERNATIONAL SEARCH REPORT

Intern. Appl. No.

PCT/US 99/19624

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N21/64 G01N21/77

According to International Patent Classification (IPC) or to both national classification and IPC

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Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 99 00663 A (DOLEMAN BRETT ; SANNER ROBERT (US); GRUBBS ROBERT H (US); LEWIS NAT) 7 January 1999 (1999-01-07) page 10, line 29 - page 11, line 9 claims 1,176  — -/-	1,3,5-7, 9,11-14

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>M C LONERGAN: "ARRAY-BASED VAPOR SENSING USING CHEMICALLY SENSITIVE, POLYMER COMPOSITE RESISTORS"</p> <p>IEEE AEROSPACE APPLICATIONS CONFERENCE. PROCEEDINGS, XX, XX, no. 3, 8 February 1997 (1997-02-08), pages 583-631, XP002078726</p> <p>page 587, left-hand column, paragraph 3</p> <p>-right-hand column, paragraph 2</p> <p>page 590, left-hand column, paragraph 3</p> <p>-right-hand column, paragraph 2</p>	1,7,13
Y		3-6, 9-12,14
Y	<p>US 5 244 636 A (WALT DAVID R ET AL)</p> <p>14 September 1993 (1993-09-14)</p> <p>cited in the application</p> <p>column 4, line 53 -column 7, line 9</p> <p>column 10, line 44 - line 56</p>	3-6, 9-12,14
A		1,7,13
A	<p>EP 0 572 157 A (PURITAN BENNETT CORP)</p> <p>1 December 1993 (1993-12-01)</p> <p>page 3, line 55 -page 4, line 59</p> <p>figure 1</p>	4-6, 10-12
A	<p>DICKINSON T A ET AL: "GENERATING SENSOR DIVERSITY THROUGH COMBINATORIAL POLYMER SYNTHESIS"</p> <p>ANALYTICAL CHEMISTRY, US, AMERICAN CHEMICAL SOCIETY. COLUMBUS, vol. 69, no. 17, 1 September 1997 (1997-09-01), pages 3413-3418, XP000720847</p> <p>ISSN: 0003-2700</p> <p>cited in the application</p> <p>page 3414, left-hand column, paragraph 3</p> <p>-page 3417, right-hand column, paragraph 3</p>	1,3,5-7, 9,11-14





## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b>  <b>G01N 33/543</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 00/16101</b>  <b>(43) International Publication Date:</b> 23 March 2000 (23.03.00)
<b>(21) International Application Number:</b> PCT/US99/20914  <b>(22) International Filing Date:</b> 10 September 1999 (10.09.99)  <b>(30) Priority Data:</b> 09/151,877 11 September 1998 (11.09.98) US  <b>(71) Applicant (for all designated States except US):</b> TRUSTEES OF TUFTS COLLEGE [US/US]; 136 Harrison Avenue, Boston, MA 02111 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> WALT, David, R. [US/US]; 4 Candlewick Close, Lexington, MA 02178 (US). MICHAEL, Karri, L. [US/US]; 77 Partridge Avenue, Somerville, MA 02145 (US).  <b>(74) Agents:</b> BREZNER, David, J. et al.; Flehr, Hohbach, Test, Albritton & Herbert LLP, Suite 3400, 4 Embarcadero Center, San Francisco, CA 94111-4187 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> TARGET ANALYTE SENSORS UTILIZING MICROSPHERES  <div data-bbox="560 1134 998 1522" data-label="Image"> </div>  <b>(57) Abstract</b>  <p>A microsphere-based analytic chemistry system and method for making the same is disclosed in which microspheres or particles carrying bioactive agents may be combined randomly or in ordered fashion and dispersed on a substrate to form an array while maintaining the ability to identify the location of bioactive agents and particles within the array using an optically interrogatable, optical signature encoding scheme. A wide variety of modified substrates may be employed which provide either discrete or non-discrete sites for accommodating the microspheres in either random or patterned distributions. The substrates may be constructed from a variety of materials to form either two-dimensional or three-dimensional configurations. In a preferred embodiment, a modified fiber optic bundle or array is employed as a substrate to produce a high density array. The disclosed system and method have utility for detecting target analytes and screening large libraries of bioactive agent.</p>		

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CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
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CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LR	Sri Lanka	SE	Sweden		
EE	Estonia		Liberia	SG	Singapore		

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/13928

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : C12P 19/34; C12Q 1/68

US CL : 435/91.2, 435/6

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/91.2, 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, EMBASE, CAPLUS, MEDLINE, LIFESCI

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,E	WO 99/31272 A1(THE GENERAL HOSPITAL CORPORATION) 24 June 1999.	1-72
Y	US 5,679,524 A (NIKIFOROV et al.) 21 October 1997, col.5, lines 55-67, col.6 lines 1-65, col. 9 lines 39-67, col.10, lines 21-38, col.11 lines 6-9.	1-10, 13-35, 38-47, 49-57, 60-72
Y	US 4,988,617 A (LANDEGREN et al.) 29 January 1991, col. 2 lines 34-68, col.3 lines 1-53, col. 6 lines 38-53, col.8 lines 39-69, col. 11 lines 45-68.	1-10, 13-35, 38-47, 49-57, 60-72
A	US 5,610,287 A (NIKIFOROV et al.) 11 March 1997.	1-72
A,P	US 5,811,239 A (FRAYNE et al) 22 September 1998.	1-72



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

01 SEPTEMBER 1999

Date of mailing of the international search report

21 OCT 1999

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/13928

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	US 5,919,626 A (SHI et al.) 06 July 1999.	1-72
Y	SCHNEIDER-STOCK et al. Improved detection of p53 mutations in soft tissue tumors using new gel composition for automated nonradioactive analysis of single-strand conformation polymorphism. Electrophoresis. 1997, Vol.18, pages 2849-2851, see entire document.	11-12, 36-37, 48, 58-59.